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CLINICAL AND EXPERIMENTAL

SEROLOGIC INVESTIGATIONS OF HERPES SIMPLEX VIRUS INFECTIONS

HARRY E. DASCOMB, M.D., CHARLES V. ADAIR, M.D., AND NANCY ROGERS, B.A.
WASHINGTON, D. C.

THE importance of the virus of herpes simplex as a human pathogen has become increasingly evident during the past decade.[1] Thus, the agent has been related etiologically to such clinical syndromes as Kaposi's varicelliform eruption,[2] fulminating visceral disease with hepatitis,[3-5] stomatitis,[6] and fatal or near-fatal encephalitis.[7] Additionally, recent investigations have shown that herpes simplex virus may be implicated etiologically in cases of aseptic meningitis about as frequently as mumps or lymphocytic choriomeningitis virus or the leptospires.[8]

The complement fixation test for infection with herpes simplex virus has provided a simple and rapid procedure for serologic investigations of this disease[9-13] which may supplement or substitute in certain instances for the more laborious neutralization test employed almost exclusively heretofore. This report is concerned with an evaluation of the complement fixation procedure for the laboratory diagnosis of herpes simplex. In addition, it presents information concerning distribution of complement-fixing antibodies against herpes simplex virus in the normal population and also data on the serologic patterns in primary and recurrent infections with the agent of this disease. In these studies, a complement-fixing antigen has been employed in which the infectivity was destroyed by exposure to ultraviolet light.

MATERIAL AND METHODS

Patients and Specimens.—The patients selected for study were U. S. military personnel or their dependents who were treated at a dispensary or hospital for herpetic or nonherpetic disease. Specimens for isolation of virus consisted of fragments of tissue, body fluids, or swabs of exudate collected from

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patients during the course of their disease or at autopsy of fatal cases. Attempts to recover the virus in animals or embryonated eggs were performed using specimens freshly collected or stored frozen at –70° C. for a few days prior to inoculation. Blood samples were obtained from the patients during the acute phase of their illness and again at variable periods during convalescence. The serum, separated from the clot, was frozen shortly after receipt and stored at –20° C. for periods of a few weeks to 7 to 8 years before being used in the present work. The majority of sera were collected less than 1 year prior to examination.

Sera for Assay of Antibody Against Herpes Simplex Virus in the Population.—Specimens of serum collected from military personnel or their dependents who sought medical care for illnesses in which the clinical and laboratory findings excluded the diagnosis of recent herpes simplex were selected as representative of the population. These sera were stored at –20° C. for periods up to 10 years, except when thawed occasionally for examination in other studies.

Serologic Tests.—

Complement fixation test: Preparation of antigen. The complement-fixing (C.F.) antigen employed in the tests consisted of the extraembryonic fluids from embryonated eggs infected with the "Z" strain* of herpes simplex virus. Unlike antigens utilized previously,[9-13] the present materials were treated with ultraviolet light to render them noninfectious. For antigen production, groups of 7- or 8-day-old embryonated eggs were inoculated via the yolk sac with 0.1 ml. amounts of infectious allantoic fluid diluted to contain $10^{2.5}LD_{50}$ of the virus. The eggs were then incubated at 35° C. and all embryos which died during the next 48 hours were discarded. All remaining embryonated eggs, both living and dead, were placed in the refrigerator 60 hours after inoculation; after being chilled for a few hours the allantoic and amniotic fluids were removed and pooled. The harvested fluid was warmed at 37° C. for 1 hour to dissolve the urates and then clarified by centrifugation at 1,500 r.p.m. for 15 minutes. The viral infectivity of the supernatant fluid was destroyed by irradiation with ultraviolet light employing a Habel-Sockrider[14] continuous flow apparatus at a flow rate of 33 ml. of fluid per minute. A Westinghouse cold cathode germicidal sterilamp 782L-30 provided ultraviolet irradiation at 2537 Å. The effluent was tested for infectivity by inoculation on the dropped chorioallantoic membrances of embryonated eggs. Prior to irradiation, the material had an infectious titer of $10^{-5.5}$ to $10^{-6.0}$. The irradiated fluid, which constituted the antigen, was not infectious. Such antigen usually exhibited a C.F. titer of 1:4 to 1:8 when assayed with 2 units of human or rabbit immune serum. Generally, the latter serum gave a slightly higher titer of antigen in simultaneous tests in which both types of serum were employed. Normal extraembryonic fluid obtained from 10- to 11-day embryonated eggs was centrifuged similarly. The nonirradiated supernatant was used as a control in the C.F. test for specificity of the antigen.

The C.F. activity of the irradiated antigen was stable for at least 6 months when the material was stored frozen at -20° C. or when dried from the frozen state. The principal complement-fixing component of the herpetic viral antigen was shown, by means of experiments employing high-speed centrifugation, to be of smaller size than the infective particles; this was in accord with the experience of others.[9, 11]

C.F. procedure: The tests were performed using a standard technique employed in this laboratory.[15] By this method, the patient's serum was inactivated at 56° C. for 30 minutes and 0.25 ml. amounts of serial twofold dilutions of the serum, covering a range from 1:4 to 1:128, were allowed to react with 2 exact units of guinea pig complement and

*Z strain herpes virus obtained from Dr. M. L. Robbins.

4 units of herpes antigen or 0.25 ml. of appropriately diluted normal control antigen. The mixtures were allowed to incubate at 4° C. for 18 hours, after which 0.5 ml. of the hemolytic system was added. The latter consisted of a mixture of 0.25 ml. of 3 per cent sheep cell suspension and 0.25 ml. of physiologic saline solution containing 3 units of sheep cell amboceptor. The tests were read after final incubation at 37° C. for 30 minutes. The titer of the serum was considered to be the highest initial dilution of serum causing complete, or almost complete, fixation of complement.

Serum neutralization test: The neutralization technique of Burnet,[16] as modified by Afzelius-Alm,[17] employing the chick embryo was used in the current work. By this method, the uninactivated serum to be assayed was diluted 1:5 in physiologic saline solution and mixed with an equal volume of herpes simplex virus suspension (FL strain*) diluted to contain 50 to 200 pock-producing doses per 0.1 ml. The mixtures were incubated at 35° C. for 1 hour, whereupon 0.1 ml. amounts were inoculated onto the chorioallantoic membranes of one-half dozen 11- to 12-day-old embryonated eggs. In certain instances, the sera were tested also at dilutions of 1:25, 1:125, and 1:625 in addition to the usual 1:5 dilution. Following incubation at 37° C. for 3 days, the inoculated chorioallantoic membranes were harvested and the pocks counted.

A serum was considered to contain neutralizing antibodies when the average pock count in test eggs was at least 75 per cent less than that of control eggs receiving mixtures of virus and normal serum.

Virus isolation technique: Autopsy or biopsy specimens were triturated in a mortar with sufficient sterile veal infusion broth to give a 10 per cent suspension and clarified by low-speed centrifugation. Penicillin and streptomycin were added to final concentrations of 1,000 units and 1,000 μg, respectively. The mixtures were inoculated intracerebrally into 1- to 2-day-old suckling mice and onto the chorioallantoic membranes of 10- to 14-day-old embryonated eggs. Specimens of blister fluid or cerebrospinal fluid were inoculated in a similar manner without preliminary processing. The development of CNS disease in mice or of pocks on the surface of chorioallantois of eggs suggested the presence of herpes simplex virus. When evidence of viral infection was absent in the original hosts inoculated, at least one additional blind passage was made before the attempt at isolation was abandoned. The recovered viruses were identified as herpes simplex by the C.F. or serum neutralization procedure using standardized antisera from rabbits or guinea pigs hyperimmunized with known strains of herpes simplex virus. Furthermore, tissues from the passage animals and eggs were shown by appropriate histologic procedures to contain typical herpetic intranuclear inclusion bodies.

RESULTS

Specificity of the C.F. Test for Herpes Simplex.—In order to determine the specificity of the C.F. test for herpes simplex, acute and convalescent sera from 52 cases of nonherpetic disease and convalescent sera from 3 cases of syphilis were tested for C.F. antibody using the ultraviolet irradiated herpes simplex antigen. The cases, which represent various types of illness, are summarized in Table I. Standard isolation or serologic procedures were used to establish the etiology of the illness in all patients mentioned in Table I, except those with aphthous stomatitis or erythema exudativum multiforme. Failure to recover virus from lesions of the latter cases was assumed to exclude herpes simplex.

It is seen in Table I that none of the patients with nonherpetic disease showed a diagnostic (fourfold or greater) increase in the amount of antibody against herpes simplex virus during the progress of his illness. However, such antibody was present at constant levels of 1:4 to 1:32 in paired sera from 25

*Supplied by Dr. Leslie Afzelius-Alm.

of the 55 cases, presumably as the result of previous infection with herpes simplex virus. None of the sera reacted with the normal control antigen, including the 3 sera from patients with syphilis. It is to be noted that sera obtained from the case of aphthous stomatitis and that of erythema exudativum multiforme showed no diagnostic rise in titer of antibody against herpes simplex virus, even though this agent has been related etiologically to such syndromes in certain instances.[18-20]

TABLE I. OCCURRENCE OF COMPLEMENT-FIXING ANTIBODIES FOR HERPES SIMPLEX IN PAIRED SERA FROM PATIENTS MANIFESTING NONHERPETIC DISEASES OF DIVERSE ETIOLOGY

CLINICAL SYNDROME	SPECIFIC ETIOLOGY	TOTAL NO. OF CASES	HERPES SIMPLEX ANTIBODY*		NO. CASES SHOWING FOURFOLD OR GREATER RISE IN HERPES SIMPLEX C. F. ANTIBODY
			PRESENT	ABSENT	
Meningoencephalitis	Mumps	20	8	12	0
	LCM	7	4	3	0
	Leptospires	4	1	3	0
	M. tuberculosis	3	1	2	0
	C. immitis	1	1	0	0
	Jap B encephalitis	1	1	0	0
	Poliomyelitis				
	Type I	3	1	2	0
	Type II	2	2	0	0
	Type III	3	0	3	0
Visceral disease					
Infectious mononucleosis	Unknown	1	0	1	0
Fever	Colorado tick fever	1	0	1	0
RMSF	*R. rickettsii*	1	0	1	0
Pneumonitis	Psittacosis	1	1	0	0
	Primary atypical pneumonia (cold agglutinin positive)	1	1	0	0
Syphilis	*T. pallidum*	3†	3	0	
Vesicular disease					
Varioliform eruption	Vaccinia	1	0	1	0
Aphthous stomatitis	Unknown	1	1	0	0
Erythema exudativum multiforme	Unknown	1	0	1	0
Total cases		55	25	30	0

*Herpes antibody when present ranges from 1:4 to 1:32 with a mean of about 16; antibody was considered absent when negative results were obtained with the 1:4 dilution.

†Single specimen only. None of these sera reacted with the normal control antigen.

Age Distribution of Antibody Against Herpes Simplex Virus.—Sera obtained from 211 military personnel or their dependents during an illness of presumably nonherpetic etiology were tested for C.F. antibody against herpes simplex virus in order to obtain information on the age distribution of this antibody and to evaluate the test as a tool for serologic survey purposes. Certain of the specimens were also assayed for virus-neutralizing capacity. The results of these tests, summarized in Table II, revealed that C.F. antibodies were present among each of the age groups of the population tested. Furthermore neutralizing antibodies were found among most of the age groups

despite the small number of sera examined. Thus, 63 of 211 (30 per cent) persons had detectable C.F. and 12 of 43 (28 per cent) had demonstrable neutralizing antibody in their sera. It is seen further that the proportion of sera with C.F. antibody was greater in the older age groups than in the younger. Thus, the values ranged from 9 to 20 per cent positive in individuals up to 14 years of age and from 25 to 75 per cent positive in older persons. The C.F. antibody in the one positive infant (less than 6 months) was probably of maternal origin since the titer was 1:16 at birth and this fell to an undetectable level by 5 months of age. The number of sera tested by the neutralization method was too small to permit valid conclusions regarding age-frequency distribution of neutralizing antibody in this group.

TABLE II. AGE-FREQUENCY DISTRIBUTION OF COMPLEMENT-FIXING AND NEUTRALIZING ANTIBODIES AGAINST HERPES SIMPLEX VIRUS

AGE GROUP	COMPLEMENT FIXATION TEST RESULTS				NEUTRALIZATION TEST RESULTS		
	NUMBER OF SERA TESTED	NUMBER POSITIVE	PER CENT POSITIVE	TITER RANGE	NUMBER OF SERA TESTED	NUMBER POSITIVE	PER CENT POSITIVE
0-6 mo.	10	1	10	16*	2	0	
7-12 mo.	7	1	14	8	1	0	
1-3 yr.	22	4	14	4-16	2	1	
4-6 yr.	30	5	17	8-32	9	2	
7-9 yr.	30	2	9	8-16	10	1	
10-14 yr.	20	4	20	4-16	2	0	
15-19 yr.	20	5	25	8-16	2	0	
20-29 yr.	32	14	44	4-32	9	5	
30-39 yr.	20	11	55	8-16	5	2	
40 yr. or greater	20	16	75	4-32	1	1	
Total	211	63	30	4-32	43	12	28

*Numbers are reciprocals of C. F. titers.

TABLE III. OCCURRENCE OF C.F. AND NEUTRALIZING ANTIBODY FOR HERPES SIMPLEX VIRUS IN SERA OF 68 NORMAL PERSONS*

NEUTRALIZATION TEST RESULT	COMPLEMENT FIXATION TEST RESULTS NUMBER OF SERA		TOTALS
	POSITIVE	NEGATIVE	
Positive	29	3	32
Negative	1	35	36
Totals	30	38	68

*Age 6 months to 40 years.

It is of importance to note that persons with C.F. antibody against herpes simplex virus usually possessed neutralizing antibody, and vice versa. This is illustrated in Table III, which summarizes the findings of the neutralization and complement fixation tests with sera from 68 normal persons. It is seen that 29 of 32 sera which contained neutralizing substance also possessed detectable C.F. antibody. Likewise, 29 of 30 sera giving positive C.F. reactions also neutralized herpes virus. A disparity occurred in only 4 of 68 sera tested by both methods. Thus, 3 of 32 sera containing neutralizing antibodies failed to fix complement and 1 of 36 sera without neutralizing antibody gave a positive C.F. result. The correlation between the results of the C.F. and neutralization tests was remarkably close considering the fact that the

two tests apparently measured antibody to different viral components, viz., soluble substance and the virus particle.[9, 11] Since the complement fixation and neutralization tests appear roughly comparable in determining past infection with herpes simplex virus,[10, 13] it seems that the C.F. method might provide a satisfactory substitute for the more laborious neutralization method employed heretofore.

Serologic Findings in 34 Patients With Herpetic Disease.—During the present investigation, laboratory observations were made in 34 cases of proved infection with the virus of herpes simplex. These patients were classified as having primary or recurrent disease on the basis of clinical history alone. Thus, the designation "primary herpetic disease" was given when the patient manifested for the first time a syndrome compatible with herpetic infection.

Serologic Findings in Primary Herpetic Disease.—Observations on 20 patients considered to have primary herpetic disease are summarized in Table IV. In each instance a significant (fourfold or greater) increase in C.F. or neutralizing antibody was demonstrable in the patient's serum during convalescence or recovery of the virus established the diagnosis of herpes simplex. The 20 patients were arbitrarily divided into four separate groups on the basis of pattern of C.F. antibody response. Group 1 consisted of 9 cases of systemic herpetic disease evidenced by fever with mucocutaneous lesions and lymphadenopathy or with central nervous system involvement. In each case, the acute phase specimen was collected early in the disease (Day 8 or less) and a rise in C.F. titer was evident during convalescence. In Cases 1 to 7, an increase in neutralizing antibodies was also detected, while in Patients 8 and 9, such antibodies were already present in the acute phase blood sample and a quantitative test was not carried out.

The patients in Groups 2 and 3 were in general similar clinically to those in Group 1 and represented several syndromes but differed in that C.F. antibody was present in the initial specimen. All patients in Group 2 developed a significant increase in C.F. antibody during the period of observation. Patient 10 showed a rise in neutralizing antibodies during his illness, and the remaining members of this group showed neutralization in both the acute and convalescent specimens. Thus, no conclusions can be drawn as to the level of neutralizing antibody present in Cases 11 to 15 since all sera were tested at a 1:5 dilution. The single patient in Group 3 was unique in that he maintained a C.F. titer of 1:16 during the period from the third to the fifty-second days after onset of aseptic meningitis, but showed an increase in neutralization antibodies between the third and twenty-sixth days.

The patients in Group 4 were of considerable interest from the serologic point of view. Each of the 4 members of the group had a mucocutaneous infection with lymphadenopathy, but none had fever, a sign which we had selected as evidence of systemic involvement. It may be noted in Group 4 that none of these patients developed complement-fixing antibodies at any time during the period of study, although herpes virus was recovered from each. However, three of the patients, i.e., Numbers 17 to 19, developed an increase

TABLE IV. SEROLOGIC AND VIRAL ISOLATION FINDINGS IN 20 CASES OF PRIMARY HERPES SIMPLEX VIRUS INFECTION

GROUP	CASE NO.	AGE	SYNDROME	DAY OF DISEASES	LABORATORY FINDINGS: SEROLOGIC C. F.	SEROLOGIC NEUT.	VIRUS ISOLATION: TISSUE EMPLOYED	VIRUS ISOLATION: RESULT
1	1.	2 yr.	Progenital vesicle, fever	2	0*	8†	Vesicle fluid	Pos.
				22	32	64		
	2.	22 mo.	Stomatitis	2	0	<5	Saliva	Pos.
				140	8	125		
	3.	29 yr.	Encephalitis	4	0	<5	Brain	Neg.‡
				6	4			
				11	64	125		
	4.	21 yr.	Aseptic meningitis	8	0	<5	None	-
				28	64	5 or>		
				92	64	5 or>		
	5.	26 yr.	Aseptic meningitis	5	0	<5	None	-
				22	16	5 or>		
	6.	11 mo.	Encephalitis	4	0	<5	None	-
				25	32	5 or>		
	7.	32 yr.	Encephalitis	2	0	<5	None	-
				12	16	125		
	8.	26 yr.	Aseptic meningitis	8	0	5 or>	None	-
				35	64	5 or>		
				150	64	5 or>		
	9.	20 yr.	Stomatitis, fever	8	0	5 or>	None	-
				23	16	5 or>		
	10.	24 yr.	Aseptic meningitis	8	32	<5	None	-
				23	128	5 or>		
	11.	30 yr.	Encephalitis following primary progenital lesion	10	8	5 or>	Brain	Pos.
				20	128	5 or>		
2	12.	19 yr.	Aseptic meningitis	5	4	5 or>	None	-
				19	16			
				42	64	5 or>		
				163	128			
	13.	19 yr.	Meningoencephalitis	6	16	5 or>	None	-
				21	32			
				57	64	5 or>		
	14.	58 yr.	Meningoencephalitis	16	8	5 or>	Cerebrospinal	Neg.
				48	64	5 or>		
				98	64	ND		
	15.	11 mo.	Stomatitis and visceral disease	18	4	5 or>	None	-
				26	16	5 or>		
3	16.	22 yr.	Aseptic meningitis	3	16	5	None	-
				26	16	25		
				52	16	ND		
4	17.	22 yr.	Oculoglandular disease	5	0	<5	Conjunctiva	Pos.
				13	0		skin	
				21	0	5 or>	vesicle	
				80	0	5 or>		
	18.	14 yr.	Conjunctivitis	11	0	<5	Swab	Pos.
				35	0	<5		
				74	0	5 or>		
	19.	30 yr.	Stomatitis adenopathy	8	0	<5	Swab mouth	Pos.
				29	0	5 or>		
	20.	36 yr.	Stomatitis adenopathy	10	0	<5	Throat washings	Pos.
				47	0	<5		

*Titer less than 1:4 the lowest serum dilution tested.

†Numbers represent reciprocals of titers.

‡Histopathologic examination, performed when patient died on the eleventh day after onset, revealed intranuclear inclusion bodies. (Report by Armed Forces Institute of Pathology.)

in neutralizing antibodies for the FL strain of virus during convalescence. To test the possibility that antigenic dissimilarity in strains[21-23] rather than differences in serologic technique might explain the disparity between the C.F. and neutralization results, the sera from Case 17 were retested with a C.F. antigen prepared from the homologous virus isolate. Here also the result was negative.

It is clearly evident, in these cases of primary herpetic disease, that neutralization tests employing but a single dilution of serum (1:5) are of limited

TABLE V. SEROLOGIC AND VIRAL ISOLATION FINDINGS IN 14 CASES OF RECURRENT HERPES SIMPLEX VIRUS INFECTION

GROUP	CASE NO.	AGE	SYNDROME	DAY OF DISEASE	LABORATORY FINDINGS: SEROLOGIC C. F.	SEROLOGIC NEUT.	VIRUS ISOLATION: TISSUE EMPLOYED	VIRUS ISOLATION: RESULT
5	21.	22	Cutaneous vesicles	3	0*	5 or>	Vesicle fluid	Pos.
			Lymphadenopathy	24	16†	5 or>		
	22.	29	Cutaneous	3	4	5	Vesicle fluid	Pos.
			vesicles	17	4			
			Lymphangitis	40	16	25		
			Lymphadenopathy fever					
			Oculoglandular	394	16	25	Swab of con-	Neg.
			disease	417	16	25	junctiva and eyelid	
	23.	51	Cutaneous bullae	1	16	125	Scrapings of	Pos.
			Fever	22	128	125	lesion	
	24.	24	Cutaneous	3	16	5	Vesicle fluid	Pos.
			vesicles	51	8	25		
	25.	22	Cutaneous	3	0	<5	Vesicle fluid	Pos.
			vesicles	13	0	5 or>		
6	26.	21	Progenital	10	8	5 or>	Swab glans	Pos.
			lesions	22	8		penis	
				42	4	5 or>		
			Recurrence:					
			Herpes	89	8		Swab glans penis	Pos.
			Progenitalis and	96	4		Swab shaft	Pos.
			stomatitis	103	8	5 or>	Swab mouth	Pos.
	27.	35	Herpes labialis	1	16		None	Pos.
				11	16	5 or>		
				25	8	5 or>		
			Herpes labialis	344	16	5 or>	Vesicle fluid	Pos.
				367	16	5 or>		
	28.	21	Progenital ulcer	21	16	5 or>	Swab ulcer	Pos.
				36	16	5 or>		
	29.	30	Cutaneous	6	32 (32)	5 or>	Vesicle fluid	Pos.
			vesicles	27	32	5 or>		
	30.	30	Cutaneous	9	16	5 or>	Vesicle fluid	Pos.
			vesicles	21	16	5 or>		
	31.	25	Cutaneous	7	16	5 or>	Vesicle fluid	Pos.
			vesicles	15	32			
				40	16	5 or>		
	32.	26	Progenital	2	64	5 or>	Vesicle fluid	Pos.
			vesicle	15	64	5 or>		
	33.	25	Cutaneous	2	32	5 or>	Skin ves.	Pos.
			vesicles	13	64	5 or>		
	34.	34	Progenital	2	32	5 or>	Vesicle fluid	Pos.
			vesicle	65	16	5 or>		

*Titer less than 1:4, the lowest serum dilution tested.
†Numbers are reciprocals of serum titers.

usefulness for routine diagnostic purpose. The value of the test was improved in a number of instances, however, when the samples were assayed by serial dilution. The quantitative complement fixation test, on the other hand, was a reliable diagnostic tool for patients with sufficient systemic involvement to result in fever, but was of no value in mild infections without fever since C.F. antibodies failed to appear following such minor illness.

Serologic Findings in Recurrent Herpetic Disease.—Fourteen patients with recurrent herpetic disease were chosen to illustrate the serologic findings in such cases. The observations are summarized in Table V. All the patients had cutaneous infection and three (Numbers 21 to 23) displayed some systemic manifestations of disease. The diagnosis of the acute illness in each patient was substantiated by the recovery of herpes simplex virus from the cutaneous lesions. Based on the serologic results, the cases were divided into 2 groups. Individuals were placed in Group 5 if there was a significant increase in C.F. or neutralizing antibody in the course of the recurrent infection. Three of the five patients in this group, i.e., Numbers 21 to 23, developed an increase in C.F. antibodies during convalescence, while two maintained constant levels of these antibodies during observation. Three patients, Numbers 22, 24, and 25, developed increased amounts of neutralizing substances and two maintained constant levels. Other investigators have shown variations in the level of C.F. or neutralizing antibodies in occasional patients observed over a period of time following primary infection,[12, 24] recurrent carrier state,[6] and experimental inoculation of herpes virus.[25] The present data on the increase of one or the other type of antibody in recurrent herpetic infection, added to the earlier observation, suggest that this phenomenon is not uncommon in those who suffer recurrent disease.

The patients in Group 6, all of whom presented evidence of localized disease without systemic involvement, had both C.F. and neutralizing antibodies in their initial specimens of serum. Furthermore, none of the patients had a significantly higher C.F. titer in his convalescent specimen. This larger group represents the usual serologic findings in recurrent herpes simplex.

DISCUSSION

The present studies of the distribution of C.F. antibodies against herpes in normal population groups of different ages provide data in general accord with the older observations of distribution of neutralizing antibodies.[26, 27] In general, the occurrence of C.F. antibodies is infrequent in children and young adults, but the majority of persons over 30 have these immune substances. While our data on neutralizing antibodies are relatively meager, they suggest that these substances also appeared later in our group than in those groups studied by others.[6, 28] It may be that the military personnel and their dependents are situated unusually favorably as regards sanitary environment and information on personal hygiene. This might account for fewer infections spread over a longer period of time and the somewhat later appearance of herpes antibodies in this population. Previous investigators,[13] in a serologic survey of herpes simplex virus infection, employing the C.F. test, reported findings which compared favorably with those obtained by neutraliza-

tion techniques. These workers also found that the C.F. antibody disappeared earlier during the postnatal period than the neutralizing substance and, furthermore, that the C.F. test was less sensitive than the neutralization technique for detecting small concentrations of antibody against herpes simplex virus. Such findings suggest that the results may be different when the two procedures are used for epidemiologic purposes. One wonders whether severe, i.e., systemic, involvement is a necessary prerequisite for the appearance of C.F. antibodies or whether recurrent minor cutaneous manifestations are sufficient to induce C.F. antibodies even though mild primary disease does not elicit these particular antibodies.

The findings in studies of primary and recurrent herpes simplex infections employing C.F., neutralization, and virus isolation methods suggested that all three techniques have a place in the laboratory diagnosis of this malady. Thus, the neutralization test is the serologic procedure of choice for the diagnosis of mild, nonfebrile, primary, mucocutaneous disease (Group 4 in Table IV), in which no C.F. antibody develops. The ease of performing the C.F. test recommends its use in primary herpetic infection with systemic symptoms, such as fever associated with central nervous system or visceral involvement or lymphadenopathy in which a rise in C.F. and neutralizing antibody occurs. A fair number of patients with recurrent herpes developed an increase in C. F. or neutralizing antibodies during convalescence. In such persons both tests were useful and essential. It is to be noted in Table V that the three patients who displayed an increase in C.F. titer (Numbers 21 to 23) suffered a more severe disease than did the two remaining patients who had only localized lesions of the fingers. All in all the findings suggest that the serologic techniques may be of assistance in the search for noncutaneous recurrent herpes simplex, for example, recurrent involvement of the central nervous system.

SUMMARY

A complement fixation test for herpes simplex employing irradiated noninfectious antigen of chick embryo origin provides a simple, rapid procedure for the specific assay of antibody against herpes simplex virus. The results of the C.F. test correlate well in the majority of instances with those of the in ovo neutralization test. However, the two procedures presumably measure different antibodies. In certain patients, namely, those with primary superficial mild herpes simplex, neutralizing antibodies appear without associated C.F. antibodies. Both types of antibodies develop in patients with severe primary herpes. In the recurrent type of disease both types are usually present in the earliest available sample of serum, but in a fair proportion of patients one or the other antibody may increase in titer during convalescence. The C.F. technique is a valuable diagnostic tool in many types of herpetic infection, especially those involving the central nervous system, but it does not replace the neutralization test or the viral isolation and identification procedure. Preliminary observations suggest that the C.F. method is useful in serologic surveys designed to detect past infection with herpes simplex virus.

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THE EFFECT OF PYRIDOXINE HYDROCHLORIDE IN ACUTE ALCOHOLIC INTOXICATION

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INTRODUCTION

THE B-complex vitamins have been the object of considerable investigation in alcoholic patients[1,2] and have, in fact, a proved utility in treating some of the complications of alcoholism. However, the evidence for the efficacy of any of the B-complex group in the treatment of acute alcoholic intoxication itself has been unimpressive. The recent work of Wordsworth[3] suggests that pyridoxine hydrochloride (B_6), when administered in intravenous doses of 50 to 100 mg., is effective in relieving the manifestations of acute alcoholic intoxication.

In a preliminary study, five patients admitted to the accident floor of the Boston City Hospital for acute alcoholic intoxication were treated with pyridoxine hydrochloride. One was given an intravenous dose of 100 mg.; two received 200 mg. each; and two were given 500 mg. of pyridoxine hydrochloride. Using the subjective criteria employed by Wordsworth, it was impossible to assess the changes, if any, occurring following injection of pyridoxine. It was clear that finer measurements were needed to assess the possible benefits of pyridoxine administration. Accordingly, certain psychological and physiological tests previously utilized by various authors[4-6] for the quantitation of alcoholic intoxication were employed to test the efficacy of pyridoxine hydrochloride.

METHODS AND MATERIALS

I. *Tests Used.*—

A. Romberg: Alterations in station and gait are among the earliest and most pronounced neuromuscular dysfunctions caused by alcoholic intoxication. Similar alterations are prominent in disorders of cerebellar function. This relationship between disorders of cerebellar function and malfunction resulting from alcohol ingestion was recognized as early as 1824,[7] and the Romberg sign, a classical test for cerebellar disorders has long been used as a test for alcoholic intoxication. In the present study, the degree of sway was estimated by inspection at frequent intervals following the ingestion of alcohol.

B. Nut-bolt Test[8]: In progressive alcoholic intoxication, neuromuscular incoordination of the upper extremities has been measured quantitatively by a variety of tests[9-11] which indicate a performance deterioration of from 10 to 100 per cent. The test used in the present study consisted in the screwing together of a ¼ inch brass nut and bolt just sufficiently to keep them joined when they were put into a nearby container. The total number of assemblies (nuts and bolts joined) completed in a two-minute period was recorded. The test was repeated at frequent intervals. (See individual charts.)

C. Flicker Fusion Frequency Test: The flicker fusion frequency[12-17] has been shown to be influenced by alcohol ingestion. Bernhard and Skoglund demonstrated that the

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flicker fusion frequency in man decreased 10 to 12 per cent after the ingestion of 100 to 150 c.c. of 80 proof whiskey. These authors further showed that alcohol has a selectively suppressing effect on the isolated negative component (P III) of the electroretinogram of the frog's eye and had an inhibitory effect on the optic nerve.

The apparatus used in the present study was designed to produce a controllable rate of flicker in a suitable light source.* The subject sat one meter from a light source of 60 foot lamberts brightness. At this distance, the flickering field had a diameter of one-half degree. Two light-dark time ratios were used: 40/60 and 95/5. Critical or threshold fusion frequencies, i.e., the rate of intermittance at which the subject ceased to detect flicker, were determined for each subject. Two threshold determinations were made for each light-dark ratio. Scores were expressed directly as the numerical critical flicker rate.

D. Johnson Code Test[18]: This test assesses a multiplicity of factors, including maintenance of vision, attention, memory, and neuromuscular coordination (for writing). The study by Johnson and Paschal revealed great individual variation; on exposure to low (12 per cent) O_2 some subjects showed a definite deterioration in performance, while others demonstrated actual improvements until just prior to collapse from low O_2. The simpler sensory motor reflexes were not noticeably impaired until the deterioration of the more highly organized responses reached an advanced stage. McFarland and Barach[19, 20] felt that this test was useful in detecting the effect of alcohol. The Johnson alphabetic code test required the subject to translate a series of 50 letters according to a prescribed alphabetic code. In the present study, 20 different tests of approximately equivalent difficulty were used. At each testing period three tests were given each subject. One and one-half minutes per test were allowed. The completed tests were scored to show the number of letters correctly translated.

E. Clinical Observations: The usual overt behavioral signs of intoxication, such as lability of mood, speech disturbances, and gross neuromuscular incoordination, were noted.

F. Blood Serum Alcohol Levels: Serum alcohol determinations[21] were made on all the alcoholic patients. Samples of blood were taken at approximately one-half-hour to one-hour intervals.

II. *Experimental Groups.*—

Two groups of subjects were studied, one consisting of five normal males (university students and medical residents) ranging in age from 23 to 34 years; the other consisted of six alcoholic patients ranging in age from 34 to 56 years, with histories of ten or more years of heavy alcoholic intake.

III. *Experimental Procedure.*—

The general experimental plan, as outlined below, was followed in all cases. Each subject was given an opportunity to familiarize himself with the tests during a period of approximately one hour, and control test observations were made. He was then given an oral dose of alcohol, in the form of 86 proof commercial whiskey, ranging from 1.25 to 3.0 Gm. per kilogram of body weight (see Table I). The various tests were run at five- to thirty-minute intervals. Intravenous injections of 0.5 to 2.0 Gm. of pyridoxine in 5 ml. of saline, or control injections of 5 ml. of saline alone, were administered to each subject one and one-half to two hours after the ingestion of the first dose of alcohol. Clinical observations were recorded throughout the experimental period.

RESULTS

The experiments on six alcoholic patients are described in detail in Table I. A general review of the findings is recorded below.

Subject H. H. (Fig. 1) was studied in three separate experiments. He was a 36-year-old man with a history of 20 years of heavy drinking. His performance was studied by the nut-bolt test, by the Romberg sign, and by clinical observations. His control scores in the nut-bolt test varied from 30 to 44 assemblies on the different days on which he was studied, but there was little

*Equipment designed by Dr. Bruce Fisher of Harvard School of Public Health.

variation during the control period on any one day. He received an initial dose of 280 ml. of whiskey in each experiment. In experiment one he received a second dose of 93 ml. of whiskey 77 minutes after his first drink. In this experiment, the number of completed assemblies fell markedly, from 32 to 14, after his second drink. He received an intravenous injection of 0.5 Gm. of pyridoxine hydrochloride 33 minutes after his second dose of alcohol, and his assembly score returned to his normal value within 45 minutes after this injection, despite a persistently elevated blood alcohol.

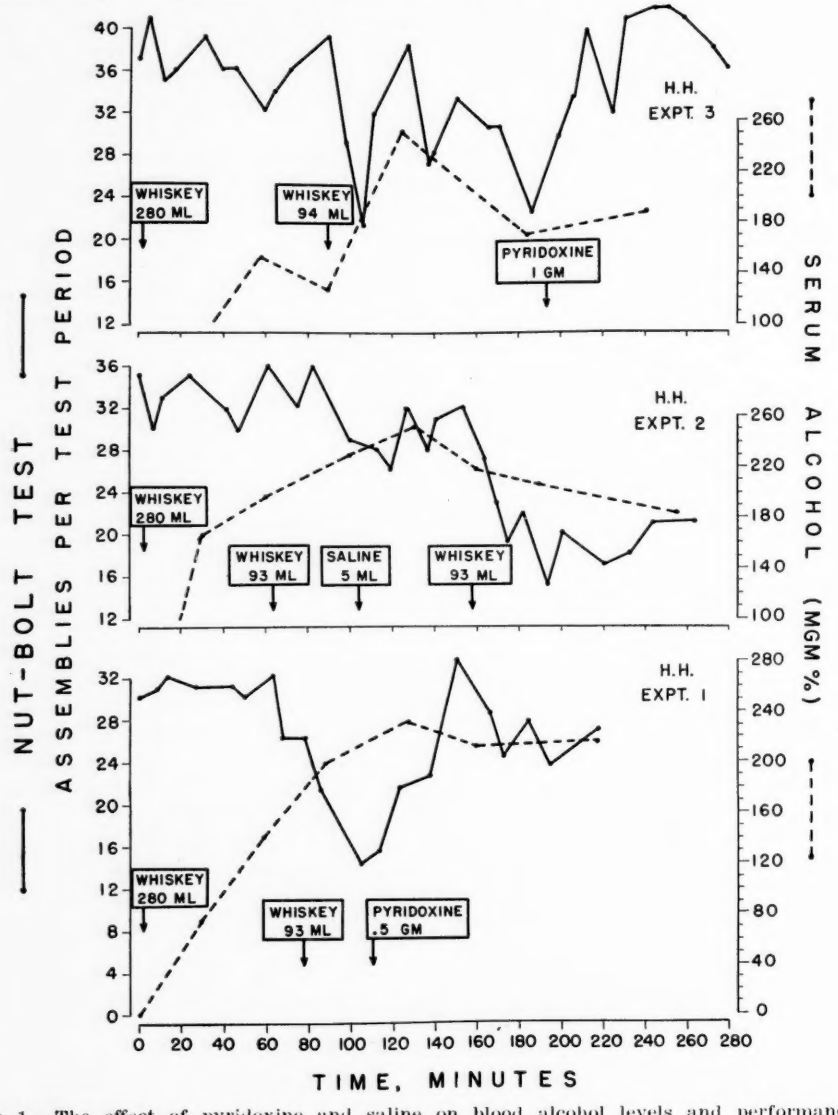

Fig. 1.—The effect of pyridoxine and saline on blood alcohol levels and performance tests during alcohol intoxication. (Subject H. H. studied on three different occasions.)

When the experiment was repeated, the expected marked fall in performance following a second dose of 93 ml. of whiskey did not occur. Despite a blood alcohol of 250 mg. per cent he assembled 32 units, which was within

the range of his control values, and he seemed only slightly intoxicated clinically. He was given an intravenous injection of 5.0 ml. of saline 40 minutes after his second dose of alcohol. There was no change in his performance following this injection. Following a third dose of whiskey, his performance fell markedly.

TABLE I. SUMMARY OF EXPERIMENTAL PROCEDURES WITH REGARD TO WEIGHT AND AGE OF PATIENT, TOTAL QUANTITY OF ALCOHOL GIVEN, PSYCHOLOGICAL TEST USED, AND TREATMENT (PYRIDOXINE OR SALINE)

		ALCOHOL			PSYCH. TEST USED				
SUBJECT	WEIGHT (KG.)	TOTAL DOSE GM./KG. BODY WEIGHT	INITIAL DOSE (ML.)	SUBSEQUENT DOSES (ML.)	NUT-BOLT	CODE	F.F.F.	PYRIDOXINE	SALINE
Chronic Alcoholics									
A. C.	74.0	2.0	324	106	X			X	
H. H. (1)*	64.0	2.0	280	93	X			X	
P. W.	54.0	2.0	237	78	X			X	
J. C.	63.0	2.5	271	91/91	X			X	
H. H. (2)	64.0	2.5	280	93/93	X				X
A. G.	76.9	2.0	316	112	X			X	
P. F.	71.9	1.5	314		X				
H. H. (3)	64.0	2.0	280	94	X			X	
Normal Controls									
F. B.	71.5	1.25	160	104		X	X	X	
P. B.	71.5	1.75	208	160		X	X	X	
N. L.	71.5	1.25	160	104		X	X		X
E. P.	70.0	1.25	104	160		X	X		X
P. T.	70.0	0.5	104			X	X		

*Subject H. H. was studied in three separate experiments.

In the third experiment, there was a distinct drop in assemblies from 39 to 21 following his second drink; but despite an increasing blood alcohol, his performance improved over a 25-minute period, and he completed 38 assemblies when his blood alcohol was at a level of 260 mg. per cent. One hundred minutes after his second drink, he was highly intoxicated, fell when performing the Romberg test, and showed a drop in assemblies, despite a falling blood alcohol level. He received an intravenous injection of 1.0 Gm. of pyridoxine hydrochloride at this time. Thirty minutes later his test performance was back to normal; he was steady on his feet, and he seemed improved clinically.

Subject A. C. (Fig. 2) was a 51-year-old laborer with a history of seven years' heavy drinking, skull fracture, and a lacerated brain. His nut-bolt assembly control value was constant at 27. One hour after ingesting 324 ml. of whiskey, he was markedly intoxicated. His test values commenced to fall at 40 minutes, falling to seven assemblies 80 minutes after his first drink. At this time he was given a second dose of 106 ml. of whiskey. Despite an intravenous injection of 0.5 Gm. of pyridoxine, which he received 20 minutes after his second drink, he remained markedly intoxicated and performed poorly in the nut-bolt test during the rest of the experiment. The expected inverse relationship between performance and blood alcohol was observed.

Subject A. G. (Fig. 2) was a 43-year-old taxi driver who had been drinking heavily for the past few years. His control values varied from 27 to 34

assemblies. His test performance, clinical symptoms, and Romberg sign remained within the normal range until after his second drink (112 ml. of whiskey 95 minutes after his initial dose of 336 ml.). He received 1.0 Gm. of pyridoxine intravenously 65 minutes after his second drink. Some clinical improvement followed the injection, but his test values did not change until more than one hour later.

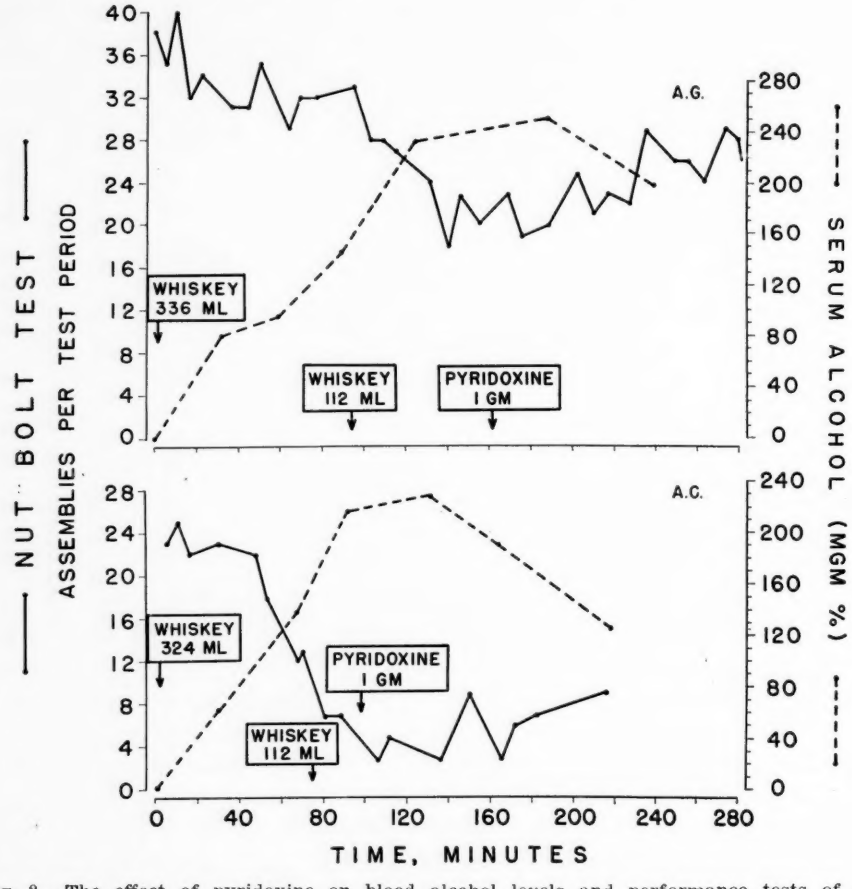

Fig. 2.—The effect of pyridoxine on blood alcohol levels and performance tests of chronic alcoholics during alcohol intoxication. (Subjects A. G. and A. C.)

Subject P. W. (Fig. 3) was a 52-year-old paper maker with a long history of chronic alcoholism. He assembled from 20 to 27 nut-bolt units in the control period. Fifty minutes after his first drink of 237 ml. of whiskey he was clearly intoxicated, his mood alternating between depression and euphoria. The variations in his test performance were attributed to his inconstant willingness to cooperate, rather than to impaired neuromuscular control. His performance fell markedly 40 minutes after his second drink of 78 ml. of whiskey. Five-tenths gram of pyridoxine intravenously at this time had no significant effect on his clinical state or on his test performance.

Subject J. C. (Fig. 3) was a 46-year-old machinist with a history of chronic alcoholism. He also had a twenty-year history of duodenal and

gastric ulcers. Partial gastrectomy was done approximately one year prior to this experiment. His control test performances varied from 28 to 33 assemblies. In only 30 minutes after the ingestion of 271 ml. of whiskey, his blood alcohol level reached a value of 206 mg. per cent. His nut-bolt assembly score at this time fell to 18 units. One hour after his first drink, his blood alcohol level fell to 184, and his nut-bolt assemblies reached a normal level of 30 units. Four minutes later he was given a second drink of 91 ml. of whiskey, and 30 minutes later his blood alcohol reached a second peak of 233 mg. per cent. He was clearly intoxicated at this time, swearing and talking incessantly, and was able to assemble only 15 units. He received an intravenous injection of 1.0 Gm. of pyridoxine hydrochloride seven minutes later. Within 15 minutes, he appeared quite sober, and his test scores were normal again, although his blood alcohol concentration was still well above 200 mg. per cent.

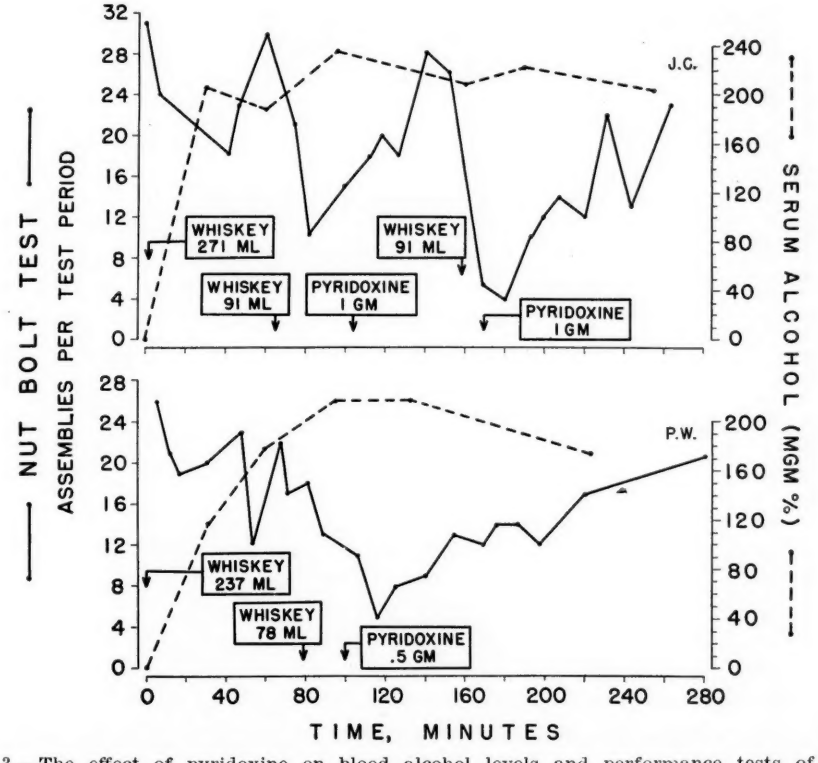

Fig. 3.—The effect of pyridoxine on blood alcohol levels and performance tests of chronic alcoholics during alcohol intoxication. (Subjects J. C. and P. W.)

He was given a third dose of 91 ml. of whiskey, followed in 10 minutes by a second injection of 1.0 Gm. of pyridoxine. Although there was some improvement in both his clinical state and his nut-bolt performance following this, the striking clinical improvement seen after the first injection of pyridoxine did not recur.

The group of normal males was studied with flicker fusion frequency measurements, the Johnson code test, and the Romberg sign, as well as the usual clinical observations (Table I and Fig. 4). Blood alcohol levels were

not determined in these subjects. The expected trend between drinking and fall in test performance was observed in all subjects in this group following their second drink. Five-tenths gram of pyridoxine was given intravenously to two (F. B. and P. B.) of the three remaining subjects, and slight improvements were noted in the performance of the men so treated; whereas, the one subject (E. P.) given saline alone failed to show any improvement and, in fact, demonstrated poorer performance in the code test (Fig. 4).

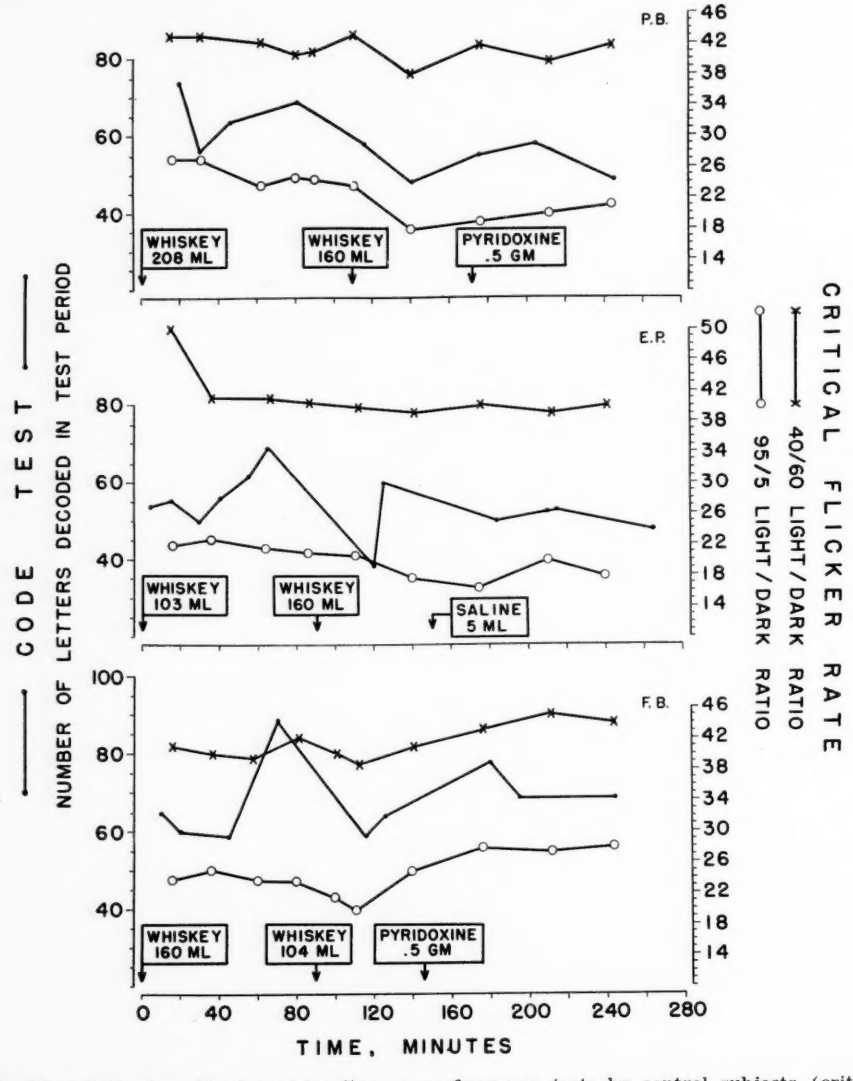

Fig. 4.—The effect of pyridoxine and saline on performance tests by control subjects (critical flicker fusion and code tests) during alcohol intoxication.

A comparison between performance values immediately before and thirty minutes after the administration of pyridoxine is made in Table II. The interval of 30 minutes was selected, as it represented the shortest interval between consecutive blood tests or consecutive flicker fusion frequency deter-

TABLE II. EFFECT OF PYRIDOXINE AND SALINE ON PERFORMANCE TESTS BY CONTROL AND CHRONIC ALCOHOLIC SUBJECTS

SUBJECT	WEIGHT (KG.)	ALCOHOL: TOTAL DOSE GM./KG. BODY WEIGHT	ALCOHOL: INITIAL DOSE (ML.)	ALCOHOL: SUBSEQUENT DOSES (ML.)	TEST	PREINJECTION: PERFORMANCE	PREINJECTION: BLOOD ALCOHOL	30 MINUTES POSTINJECTION: PERFORMANCE	30 MINUTES POSTINJECTION: BLOOD ALCOHOL
A. C.	74.0	2.0	364	106	N-B[1]	7	2.14	3P*	2.20
P. W.	54.0	2.0	237	78	N-B	13	2.14	9P	2.12
H. H. (1)	64.0	2.0	280	93	N-B	14	1.96	22P	2.26
J. C.	63.0	2.5	271	91/91	N-B	15	2.33	28P	2.18
J. C.					N-B	5	2.07	12P2	2.17
H. H. (2)	64.0	2.5	280	93/93	N-B	29	2.31	28S†	2.42
H. H. (3)	64.0	2.0	280	-	N-B	22	1.75	31P	1.90
A. G.	76.9	2.0	336	112	N-B	21	2.32	20P	2.51
F. B.	71.5	1.25	160	104	Code	64	-	78P	-
P. B.	71.5	1.75	208	160	Code	49	-	59P	-
E. P.	70.0	1.25	104	160	Code	60	-	50S	-
						95/5	40/60	95/5	40/60
F. B.					F.F.F.[2]	25.2	40.6	28.2P	43.5P
P. B.					F.F.F.	19.0	42.0	19.7P	40.0P
E. P.					F.F.F.	17.5	39.2	16.2S	39.8S

[1]Nut-bolt Test.

[2]Flicker Fusion Frequency Test.

*P = Pyridoxine; P2 = Second dose of pyridoxine given during the course of the experiment.

†S = Saline.

minations. The standard deviation of improvement was 7.4 about a mean of improvement of 5.6. With such a wide spread around the mean, further statistical analysis would be of little value.

The code test performance scores for the entire group followed a rather consistent pattern. Low performances occurred at 30 to 45 minutes after the first drink and 20 to 30 minutes after the second drink. In contrast, high performance values were obtained at 60 to 88 minutes following the first drink and 30 to 75 minutes after the second drink.

DISCUSSION

The state of alcoholic "intoxication" is a complex of psychic and organic alterations, and the assessment of a drug for the treatment of this syndrome is most difficult. Much work[4, 5, 6, 18] has been done to quantitate intoxication on a neuromuscular level, and some attempts[4, 18] have been made to assess changes in higher cortical function; but behavioral, attitude, and mood alterations remain as important but unevaluated components. Our results, therefore, must be viewed with full knowledge of the limitations of such measurements.

The variations in performance which did not correlate with serum alcohol levels or the apparent clinical state of the subjects may well be attributed to the psychic changes—attitude and cooperativeness—for which we have no measure. Further, our serum alcohols, determined as they were at half-hour to hour intervals, permitted only an approximation of trends rather than an exact description. It is clear, however, from the recorded results that whatever effect pyridoxine may have, it is not sufficiently great to make it immediately and strikingly obvious, as suggested by Wordsworth.[3] Swank[25] and oth-

ers, working on the effect of pyridoxine deficiency, have demonstrated definite neurological lesions pathologically. These lesions, this group feels, are not reversible. There is no question in view of this work and the work of Molony,[22] Coursin,[23] and Hunt[24] that pyridoxine is important in the maintenance of a normal nervous system. However, there is no rationale in this work for an assumption of an instantaneous effect on alcoholic intoxication.

CONCLUSION

No prompt or consistent effect of intravenous pyridoxine on alcoholic intoxication was noted.

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SERIAL URINARY ADDIS COUNTS IN PATIENTS INOCULATED REPEATEDLY WITH POLIOMYELITIS VACCINE PREPARED FROM MONKEY KIDNEY TISSUE CULTURES

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EARLIER studies[1, 2] have indicated that one or more inoculations of poliomyelitis vaccine necessary to produce an antibody response resulted in no clinically discernible untoward effects. Although no suggestion of adverse effects upon the kidney has been observed clinically in human subjects, or histologically in monkeys[3] inoculated with poliomyelitis vaccine from fluid derived from cultures of monkey kidney tissue, it seemed appropriate to investigate this question further. It is of interest to recall that preparations such as liver extract and insulin, and several glandular extracts, which also are derived from mammalian organs, have had widespread use and appear not to have produced damage or sensitization in the corresponding organ tissue. Although, under certain conditions, tissues such as central nervous system[4] and testis[5] appear capable of inducing tissue damage directly when administered as antigens, we are unaware of any studies that have shown the production of renal damage in either homologous or heterologous species following injection of kidney tissue suspensions alone. The report of the production of renal damage by the Caveltis,[6] using mixtures of kidney tissue suspension and bacterial antigens, has not been confirmed by several investigators.[7, 8] Nevertheless, damage to kidneys, apparently on the basis of an immunologic reaction, can readily be induced experimentally by the injection in one species of a "nephrotoxic" antiserum from a heterologous species,[9-11] as in the case of renal lesions produced in rats by the injection of specific antiserum from rabbits inoculated with rat kidney tissue. However, these reports make no mention of kidney lesions attributable to the injection of renal tissue alone in the immunized animals.

It should be noted that in studies with central nervous system and testis, as well as in the studies with kidney plus bacterial antigens by the Caveltis, relatively concentrated suspensions of tissue have been used as the antigens to produce organ tissue sensitization. The vaccine currently employed is prepared from fluid derived from cultures of monkey kidney tissue, the cells of which are destroyed by virus cytopathogenic effect; the harvested fluid medium, which is very low in soluble protein, is further treated by passage through bacteria-tight filters.[12] Thus, any cell or tissue components that

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might be thought to be capable of producing undesirable effects would have been eliminated or would necessarily be either soluble or of particle size that would be small enough to pass through bacteria-withholding filters.

From the above considerations it would seem unlikely that a vaccine of the kind described could produce renal sensitization, nor, in fact, has it as yet been observed. Nevertheless, a more intensive clinical study in human subjects was undertaken; this paper reports the results of serial Addis counts and other clinical evaluations of kidney function in a group of 14 children who received 3 or 4 doses of poliomyelitis vaccine over a period of three months.

METHOD OF STUDY

Selection of Patients.—Fourteen patients, all males 6 to 15 years old, were chosen for the study. All cases were patients at a convalescent home* for crippled children. The original disease in 11 patients was paralytic poliomyelitis of 2 to 12 months in the convalescent stage, and with involvement of one or two limbs. The 3 remaining cases were patients with cerebral palsy of moderate severity which limited them to wheel-chair activity. At the time the study was initiated, none of the 14 patients was totally immobilized by illness. Past medical histories of all cases were reviewed with particular reference toward exclusion of previous urinary tract disease. The poliomyelitis patients had not been catheterized during their acute illnesses.

Prior to inoculation with vaccine all patients received a complete physical examination; x-ray films of the chest and abdomen were taken, and blood was drawn for control hematologic and biochemical determinations. In addition, phenolsulfonphthalein (PSP) excretion tests, several routine urinalyses, as well as a variable number of Addis counts were performed before vaccine was given. Beginning several weeks before inoculation with vaccine and during the entire investigation rectal temperatures were taken daily in the morning and evening.

Technique of Addis Counts.—Cells in urine sediments were counted in a standard manner[13] after the technique originally described by Addis.[14] Urine collections were always overnight specimens over a recorded time interval, but not necessarily over a 12-hour period. However, all counts were calculated and expressed in terms of numbers of cells per 12-hour excretion. A classification of 3 cell types was employed: red, white, and "other" cells. Two separate counts were made from each specimen for a final average value. All counts were made by only two workers (S. Z. and F. N.).

Qualitative tests for urine albumin were made by boiling the supernates of centrifuged urine for 5 minutes in the usual manner.

Vaccine Used.—The poliomyelitis virus vaccine used was a single preparation (Trivalent Poliomyelitis Vaccine No. 19) prepared from monkey kidney tissue culture fluid containing the Mahoney, MEF-1, and Saukett strains representing virus types I, II, and III, respectively. The culture fluid medium consisted of synthetic mixture 199[15] and also contained streptomycin and penicillin, in an original concentration of 250 and 500 units, respectively. The vaccine was prepared from culture fluids† by treatment with 1:4,000 formalin as previously described.[12] This lot of vaccine had a total nitrogen content of 0.21 mg. per milliliter and a protein nitrogen of 0.008 mg. per milliliter.

Schedule of Vaccine Inoculations.—Twelve of the 14 patients received a total of four 1 ml. doses of poliomyelitis vaccine and two were given three doses, according to the schedule in Table I.

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*Industrial Home for Crippled Children, Pittsburgh, Pa. The generous cooperation of the following is gratefully acknowledged: Mrs. E. deMey, Superintendent; Dr. J. A. Heberling, Chief of Staff; Miss B. Grierson, Social Service; Miss M. Bruce, Chief Nursing Supervisor; Nursing Supervisors Mrs. S. Jones and Mrs. R. Leffler; and others who assisted.

†Produced at Connaught Laboratories, Toronto, Canada.

TABLE I

NO. OF PATIENTS	INTERVALS BETWEEN DOSES IN DAYS		
	1ST & 2ND	2ND & 3RD	3RD & 4TH
6	11	10	21
3	9	11	21
2	11	15	20
1	11	24	21
2	9	11	

Three of the patients who received four doses of poliomyelitis vaccine and two of those given three doses were first given one or two injections of diphtheria-pertussis-tetanus (DPT) fluid vaccine nine days apart for control purposes. Both the DPT preparation (Tridipigen*) and the poliomyelitis vaccine were administered intramuscularly in doses of 0.5 ml. and 1.0 ml., respectively.

RESULTS

Serial Addis Counts.—In all patients, during the study, only occasional casts were observed, and there was no change in numbers as compared to those observed during the prevaccine injection period; therefore, these urinary elements will not be considered further. The number of Addis counts done on each patient and their time relationship to vaccine injections is presented graphically in Fig. 1. Counts of either red, white, or "other" cells which exceeded one million per 12-hour excretion period were differentiated from counts less than one million per 12 hours and are designated in Fig. 1 by appropriate symbols. In those patients whose Addis counts remained less than one million cells during the entire study, no definite patterns of cell counts in association with vaccine injections were apparent. No statistical analysis of the cell counts was attempted.

As can be seen from Fig. 1, six of the fourteen patients were found to have one or more Addis counts which exceeded one million red, white, or "other" cells during the 3-month study period. Although these findings appear not to have been related to the inoculations, further details in each instance are presented. In three cases there were only one or two isolated Addis counts of white cells which exceeded one million (Patients 7, E. C., 13, D. C., and 14, J. S.). In one patient (No. 5, E. K.) counts of 1.1 to 2.3 million white cells were observed on three occasions after the last dose of poliomyelitis vaccine. However, counts of similar magnitude were observed in this patient twice after injection with DPT, before poliomyelitis vaccine was received. His specimens also showed spermatozoa quite frequently.

In Patient 2 (W. E.), a count of 3.3 million red blood cells occurred six days after the last injection of poliomyelitis vaccine, but further study was delayed one week because the patient had been discharged home. Subsequent counts disclosed one specimen of 2.2 million white and a similar number of "other" cells. These were associated with two urine cultures positive for small numbers of *Staphylococcus albus* and alpha streptococci, respectively, as well as the presence of Gram-positive cocci in smears of cultured urine sediments. Occasional masturbation was admitted by the patient.

*Kindly supplied through the courtesy of Eli Lilly and Company.

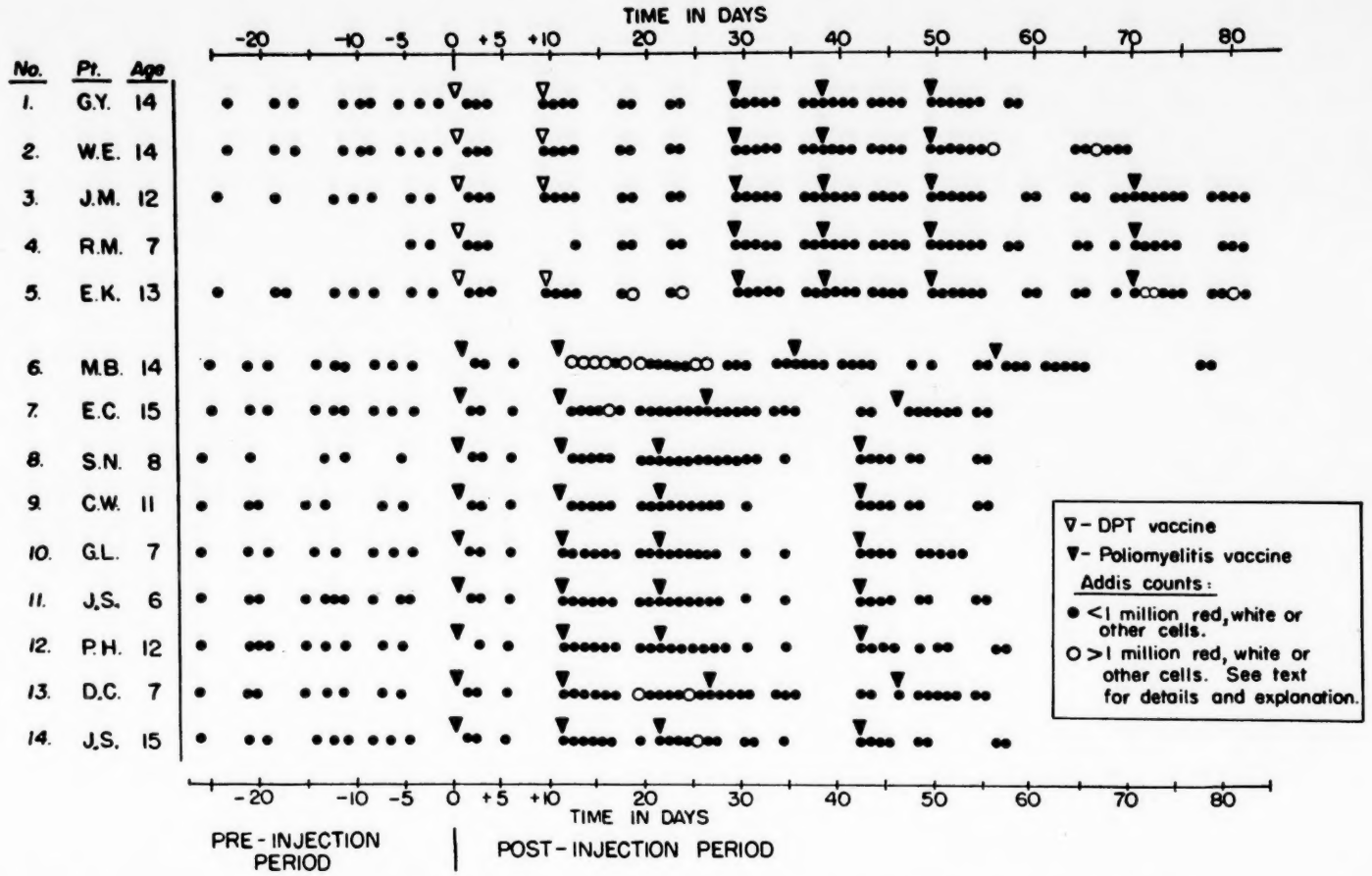
TIME IN DAYS
-20 -10 -5 0 +5 +10 20 30 40 50 60 70 80
No. Pt. Age
1. G.Y. 14
2. W.E. 14
3. J.M. 12
4. R.M. 7
5. E.K. 13
6. M.B. 14
7. E.C. 15
8. S.N. 8
9. C.W. 11
10. G.L. 7
11. J.S. 6
12. P.H. 12
13. D.C. 7
14. J.S. 15
▽ - DPT vaccine
▼ - Poliomyelitis vaccine
Addis counts:
● <1 million red, white or other cells.
○ >1 million red, white or other cells. See text for details and explanation.
-20 -10 -5 0 +5 +10 20 30 40 50 60 70 80
TIME IN DAYS
PRE-INJECTION PERIOD
POST-INJECTION PERIOD

Fig. 1.—Number of urine Addis counts and time relationship to vaccine injections in 14 patients.

Patient 6 (M. B.) showed an increase in red, white, and "other" (unidentified) cells after the second injection of poliomyelitis vaccine. No urines had been examined for a period of six days prior to this injection. Maximum counts of 5.45 million red and 1.41 million white cells on day 13, and 4.29 million unidentified cells on day 14 of the study period were observed. Trace albumin reactions were present also and persisted for 7 days. After 7 days, all three cell types returned to values of less than one million, except for days 25 and 26 when 1.1 million unidentified cells were recorded. During the period of increased cell counts, a urine culture was sterile, physical examination was unchanged, no symptoms were present, and clinical laboratory tests were normal. The patient admitted occasional masturbation. The unidentified cells present in M. B.'s urine presented an unusual appearance. They were large with considerable morphologic variation and often appeared to contain granular cytoplasmic material. By examination of stained preparations the nature or origin of these cells could not be determined.* During and after the third and fourth injections with poliomyelitis vaccine, frequent Addis counts were done and all remained well below one million for all types. All further urine albumins were negative.

OTHER CLINICAL LABORATORY STUDIES

PSP Excretion.—Fractional PSP tests by the intravenous route were performed on each patient during the control observation period and again within 5 to 10 days after the last injection with poliomyelitis vaccine. Before receiving vaccine, all patients except one showed PSP excretion of at least 25 per cent in the first 15 minutes and/or 50 per cent in 60 minutes. The exceptional case had 20 per cent excretion of dye in 15 minutes, but a total of 60 per cent in 60 minutes. Postvaccine tests showed no significant differences from results obtained before administration of vaccine.

Urine Volume and Specific Gravity.—Since no attempt was made to maintain a constant daily fluid intake and because time intervals of overnight urine collections were not controlled, there was considerable variation in the volumes of urine collected for a given patient. No particular trends or unusual changes in either urine volumes or specific gravity were observed during the study.

Hematologic and Chemical Determinations.—Hemoglobin, white blood count, and sedimentation rate remained unchanged in all patients after the series of poliomyelitis vaccine injections were completed.

In each case blood nonprotein nitrogen, sugar, and serum calcium, total inorganic phosphorus, total protein, albumin, globulin, sodium, potassium, chloride, and bicarbonate were determined† from fasting blood specimens. Values were normal in every instance during the control period and again within 5 to 10 days after the last dose of vaccine.

*We wish to thank Dr. Frank J. Dixon, Jr., and Dr. George H. Fetterman for their examination of slides of the urinary sediment.

†We are indebted to Dr. T. S. Danowski in whose laboratory these determinations were performed.

Neutralizing Antibody Levels.—Neutralizing antibody titers were determined* for each patient before poliomyelitis vaccine was given and at various intervals before and after later doses of vaccine. Several patients who already possessed antibody prior to receiving vaccine exhibited a booster type of response from the first injection, while a lower, more gradual response was demonstrated in those without pre-existing antibody. In all patients serial serologic tests showed that the poliomyelitis vaccine used produced substantial rises in antibody titer.

DISCUSSION

Urinary findings commonly associated with experimental renal sensitivity in animals[11] and glomerulonephritis in man[16] are albuminuria, cylinduria, and hematuria. Similar changes, especially albumin and casts, in the urine usually accompany the damage that results from a variety of kidney injuries, such as acute mercury poisoning,[17] carbon tetrachloride intoxication,[18] and shock.[19] Therefore, it is reasonable to expect that if renal sensitization or other injury might occur as a result of multiple injections of poliomyelitis vaccine, it would manifest itself chiefly in the appearance of albumin, casts, and red blood cells in the urine.

Under the conditions of this study, there was no evidence to indicate that repeated administration to children of poliomyelitis vaccine prepared from monkey kidney tissue cultures produced renal damage or sensitization. In five of the six patients who showed one or more elevated Addis counts during the entire investigation, such results were either isolated or sporadic events and could not be attributed to the vaccine injections.

The unusual cells seen in the urine of Patient 6 (M. B.) appeared, in certain respects, to resemble the description of the granular motility cell studied by Jackson[20] and referred to by Sternheimer and Malbin.[21] The latter considered these cells as being characteristic of pyelonephritic urine, whereas the former author believed that such cells represent polymorphonuclear leukocytes undergoing degeneration from nonspecific damage, including hypo- or hypertonicity. In this connection, it is of interest that M. B. always had a urine of high specific gravity. There was no supporting evidence to interpret the transient period of elevated Addis counts in this patient as a manifestation of injury or damage to the kidney. Masturbation must be questioned as a possible cause for the change in Addis counts. The failure to reproduce another episode of increased Addis counts with two additional injections of poliomyelitis vaccine is strong evidence that the phenomenon was not due to some type of renal sensitization.

The observations reported are limited in respect to numbers of subjects studied as well as to the period of time involved. Attention was directed primarily toward the possible occurrence of organ sensitization detectable clinically by intensive serial observation. Obviously, only long and extensive experience can provide conclusions beyond the scope of this study.

*Neutralization tests were done by Miss Elsie Ward.

SUMMARY

Fourteen hospitalized patients who received repeated injections of poliomyelitis vaccine prepared from monkey kidney tissue cultures were studied by serial Addis counts and clinical observation.

There was no evidence from this investigation of the occurrence of clinical manifestations of kidney damage or injury. Unusual-appearing cells which could not be identified were noted in the urine of one patient, but their occurrence could not be attributed to the vaccine. Other clinical and laboratory observations in the group of patients studied revealed no evidence of untoward effects.

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LABORATORY FINDINGS IN EPIDEMIC HEMORRHAGIC FEVER

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IN 1951, United Nations physicians in Korea encountered a disease previously unknown to them, epidemic hemorrhagic fever.[1] Clinical and laboratory research into the problems of the disease was started at various United States Army medical installations. In 1952, these efforts were co-ordinated at the "Hemorrhagic Fever Center" in Korea. This report presents an over-all description of hematologic, urinary, and blood chemistry findings in a group of 76 patients, including those admitted consecutively with illness of less than 60 hours' duration and including mild, moderate, severe, and fatal cases as they occurred at random. In order to study the prognostic value of this laboratory information, a comparison is made between data from the group of 76, 92 per cent of whom survived, and data from a group of 22 patients who died (6 fatalities of the original 76 consecutive cases plus 16 others).

PROCEDURES AND METHODS

The following examinations were performed on daily samples of the peripheral blood of 76 patients: white, differential, and platelet cell counts; blood urea nitrogen, creatinine, hemoglobin, and hematocrit determinations; and erythrocyte sedimentation rates. Daily urine specimens of 69 of these patients were examined for specific gravity and presence of protein. The following studies were performed on specimens from fewer than 69 patients: direct peripheral blood eosinophil counts,[2] modified Addis counts on 12-hour urine specimens,[3, 4] thymol turbidity tests,[5, 6] cephalin-cholesterol flocculation tests,[7] serum bilirubin levels,[8] urine bilirubin values,[9] urine urobilinogen contents,[10] whole blood uric acid values,[10] serum alkaline phosphatase activity,[10] and fasting blood sugar levels.[11] Sternal marrow was examined on or about the fourth, tenth, seventeenth, and twenty-sixth days of illness in 11 patients; and 21 marrows were examined at autopsy. An analysis was made of the available laboratory data in 22 patients who died of the disease.

Smears of peripheral blood were stained with Wright's stain, and each differential white blood cell count was based on the examination of 250 cells. Eosinophils and platelets were stained with phloxine and Rees-Ecker solutions,[2, 12] respectively. Using oxalated whole blood, urea nitrogen was determined by the urease method[10] and creatinine by the creatinine-zinc chloride technique.[10] Hemoglobin content and hematocrit were determined by the copper sulfate specific gravity procedure[13] and erythrocyte sedimentation rate by the Wintrobe method.[14]

The presence of protein in the urine was detected by the addition of 20 per cent sulfosalicylic acid, and the amount of protein was expressed as ranging from negative to 4 plus. Modified Addis counts were performed on 12-hour (4 P.M. to 4 A.M.) urine collections. A 15 ml. aliquot was centrifuged at 1,800 revolutions per minute for 30 minutes,

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From the Hemorrhagic Fever Center, Korea, of the United States Army and from the Departments of Preventive Medicine and Medicine, School of Medicine, Western Reserve University, and the University Hospitals, Cleveland, Ohio.

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and the supernatant urine was decanted. The sediment was suspended in normal saline to a final volume of 1 ml. and stained with a gentian violet, safranin O mixture.[15] The formed elements in the 0.9 c.mm. volume of a blood cell counting chamber were counted, and the number of formed elements per 12-hour specimen was calculated from the following formula:

$$\frac{(\text{12-hour volume})\ (\text{number of cells})\ (1.1)\ (1000)}{15} = \text{total number of cells per 12-hour specimen}$$

RESULTS IN 76 PATIENTS

Epidemic hemorrhagic fever among the 76 patients was characterized by the presence of febrile, gastrointestinal, cardiovascular, hemorrhagic, renal, and neurologic manifestations. There was marked variation in the type, duration, and severity of manifestations, and some cases were so mild that diagnosis was made with difficulty while other cases terminated in death within a few days. Severe involvement usually included hemorrhage, shock, and renal failure. Fig. 1 illustrates the general chronologic relationship of clinical aspects subdivided according to the pathologic physiology of the disease, and the figure will serve the reader as a graphic point of reference in considering the laboratory data to be presented.

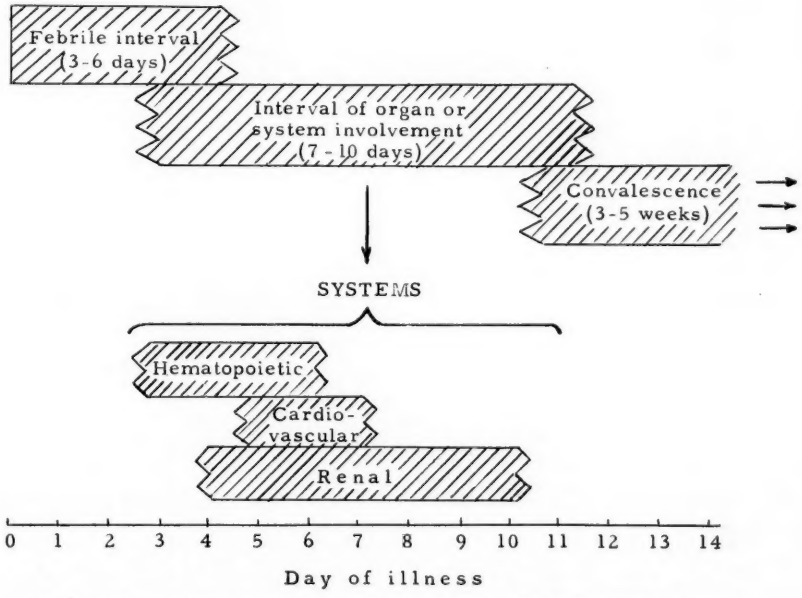

Fig. 1.—Chronology of clinical aspects of epidemic hemorrhagic fever.

A. *Febrile Interval.*—As a rule, disease started abruptly with headache, chills, and fever. For the first three to six days, signs and symptoms included diaphoresis, thirst, malaise, fatigue, eye pain, photophobia, blurred vision, coryza, nasal congestion, cough, backache, myalgia, arthralgia, facial flush, mucous membrane injection, conjunctival injection, generalized lymphadenopathy, and such gastrointestinal manifestations as anorexia, nausea, vomiting, and abdominal pain. Peripheral blood white cell counts were generally normal between the first and third days of illness and increased to a maximum

about the sixth day of illness. The highest white cell counts for each patient ranged between 9,000 and 60,000 cells per cubic millimeter with counts of 20,000 or more in 23 patients. Of the maximum counts, 90 per cent occurred during the first week of illness. An initial leukopenia (5,000 cells or less) was observed for one to three days in 20 patients, but leukocytosis developed in all 20 within the first week.

Differentiated myelocytes, metamyelocytes, stabs, atypical lymphocytes,[14] plasma cells,[14] Türck irritation cells,[14] and atypical monocytes were observed in the peripheral bloods of all 76 patients studied. In most instances, initial smears revealed abnormally increased percentages of granular leukocytes, the percentages decreasing to normal about the sixth day of illness. Inversely, the relative and absolute number of cells in the lymphoid series increased and reached a peak about the sixth day of illness. Monocyte percentages, though within normal limits, were often increasing at the same time, and occasional promonocytes and bizarre histiocytoid cells were noted.

The appearance of differentiated myelocytes, metamyelocytes, and stab cells between the first and third days of illness constituted a leukemoid response that was generally maximal on the third or fourth day. Stabs and metamyelocytes were observed in the early blood smears of all 76 patients, while myelocytes occurred in 62 of the 76. At the same time, toxic granulation was present in many neutrophils. Lymphoid cells were classified on the basis of nomenclature used in American medical literature, namely: atypical lymphoid cells after Downey,[16] plasma cells,[14] and Türck irritation cells.[14] This was in contrast to the use of the terms "plasma cells" by the Japanese[17] and Türck cells by the Russians[18] to include all atypical cells of the lymphoid series. In the series of 76 patients, cells of the lymphoid series that were noted included mature lymphocytes, type I cells after Downey,[16] type II cells after Downey,[16] type III cells after Downey,[16] plasma cells, and Türck irritation cells.[14] Of the atypical lymphoid cells, Downey type I cells predominated. Plasma cells occurred in the blood of 60 of the 76 patients, and only occasional Türck irritation cells were observed.

During the acute febrile phase, the erythrocyte sedimentation rate (Wintrobe) was generally normal. In all patients, it was increasing toward the end of the first week of illness, and in a few it was increased on admission.

B. *Interval of Organ or System Involvement.*—Manifestations of organ or system involvement became apparent between the third and fourth days of illness, primarily in the hematopoietic, cardiovascular, and renal systems. This phase started while fever was still present and persisted for a week or more beyond the acute febrile interval. During this time, total peripheral blood white cell counts were decreasing, and the leukemoid response disappeared. Although the percentage of lymphoid cells was usually decreasing from its peak level, atypical cells of this series persisted; and in many patients, monocyte percentages increased during the second week of illness. A secondary leukocytosis of lesser degree than the original was commonly observed at the end of the second week, and the erythrocyte sedimentation rate was usually increased during the second week.

Increased percentage of eosinophils (6 to 11 per cent) occurred in the peripheral blood smears of 22 of the 76 patients, and these increases were observed between the fourth and tenth days of illness in 19 instances. Absolute eosinophil counts in peripheral blood, however, were within normal limits (50 to 300 cells per cubic millimeter) in 40 patients in whom such counts were performed daily for 15 days. No significant change in the number of basophils was demonstrated during the course of the disease.

1. *Hematopoietic involvement:* About the third day of illness, hemorrhagic manifestations became apparent. Petechiae, purpura, ecchymoses, epistaxis, hemoptysis, hematemesis, melena, and hematuria were among the common bleeding phenomena. Although bleeding was a frequent occurrence, it was usually small in amount and only rarely resulted in any decrease in peripheral red blood cell count. Nucleated red blood cells (0.4 to 4 per 100 white blood cells) were observed for one to three days in the peripheral blood smears of 11 patients at this time.

Platelets were decreased in number to less than 220,000 per cubic millimeter (normal of 250,000 to 400,000) for an average of four days (range of one to ten days) in 68 of the 76 patients. Forty-six patients had counts below 150,000 per cubic millimeter, and 25 had counts of less than 100,000 per cubic millimeter. The lowest platelet counts occurred between the first and ninth days of illness, 90 per cent occurring between the second and sixth days. The number of platelets increased to normal levels between the fifth and sixteenth days of illness, and 90 per cent of the returns to normal occurred between the sixth and tenth days. At this time, many of the counts were transiently increased to levels as high as 570,000 per cubic millimeter. In 74 patients, one to nine macroplatelets (larger than 7 microns in diameter) per 100 white blood cells were present for one to eight days (average of 3 days) between the first and sixteenth days of illness. In 92 per cent of the positive examinations, the macroplatelets were observed between the second and tenth days of illness.

In a series of 11 patients, 33 specimens of sternal bone marrow were examined. Of these, the marrow was normal in four patients who had a total of 13 marrow examinations between the second and twenty-eighth days of illness. In six patients, nine marrow specimens obtained between the second and twenty-fifth days of illness revealed a decreased myeloid:erythroid ratio. One patient had an increased myeloid:erythroid ratio on the eighth day of illness. The remaining ten marrow smears from seven patients were normal.

In relation to the hemorrhagic phase, it should be noted that increased capillary fragility and prolonged bleeding time have been previously demonstrated in epidemic hemorrhagic fever.[19] These aspects were not studied in the present series of patients.

2. *Cardiovascular involvement:* Between the third and sixth days of illness, evidence of cardiovascular involvement included such clinical findings as capillary dilatation, decreased peripheral vascular resistance, blood pressure lability, and occasional electrocardiographic abnormalities. When this involvement was severe, a hypotensive or shock phase resulted, lasting 12 to 72 hours and often associated with the presence of increased capillary perme-

ability, edema, and hemoconcentration. Most of the deaths during the early course of epidemic hemorrhagic fever have been noted to occur during the shock phase.

Laboratory findings associated with the hypotensive phase included abnormally increased hemoglobin contents and hematocrit values. In 65 patients, increased hemoglobin contents of 16.6 to 20.3 Gm. per 100 milliliters occurred between the second and eighth days of illness. In 59 patients, increased hematocrits of 49 to 60 per cent occurred during this same interval.

3. *Renal aspects:* Between the third and sixth days of illness, renal disease became apparent and resembled "lower nephron nephrosis" with such findings as albuminuria, oliguria, edema, azotemia, fixed urine specific gravity, hypertension, and occasional uremic manifestations. A spontaneous diuresis began between the seventh and eleventh days of illness, after which time clinical signs and symptoms usually disappeared rapidly. During the subsequent convalescent phase, renal function returned to normal (as did the erythrocyte sedimentation rate and peripheral white blood cell count). Neurologic manifestations were often associated with the presence of severe uremia.

The volume of urine output in 69 patients studied was generally less than 1,000 ml. on the second to fifth days of illness and more than 2,800 ml. on the tenth to seventeenth days (intervals of oliguria and diuresis, respectively). In 46 of these patients, urine outputs of 0 to 550 ml. were noted for one to eight days (average of two days), and these low outputs were recorded between the second and eleventh days of illness (95 per cent between the second and eighth days). Daily urine outputs of 3,000 to 6,800 ml. were noted in 39 of the 69 patients for one to seven days (average of three days), and these outputs occurred between the fifth and seventeenth days of illness.

All of 69 patients studied had proteinuria ranging from a trace to 4 plus. The degree of proteinuria was 3 to 4 plus in 58 patients and was observed to be 4 plus for a total duration of one to eight days (average of three days) between the second and thirteenth days of illness. Ninety-two per cent of the 4 plus urines occurred between the third and eighth days. Urine specific gravity was generally greater than 1.020 between the second and fourth days of illness and fell to 1.010 by the tenth day.

The number of red cells, granular cells, and casts in the urine as shown by modified Addis counts was increased at some time during the disease in the 25 patients so studied. Mean counts of white cells and epithelial cells in this group of patients were within normal limits. Increased numbers of red cells occurred on the third to sixth days of illness (maximal on the fourth day). "Granular cells" as described by Ganong and associates[20] were seen in the urines of 24 of the 25 patients and were most abundant on the fifth day of illness. The typical cell was globular, varied from 15 to 50 microns in diameter, had a doubly refractile wall, contained vacuoles, and had a degenerated nucleus which was sometimes pyknotic. Unidentified inclusion bodies were

occasionally observed in the smaller cells of this type. The cells were stained by Wright's or Giemsa stains after being fixed in methyl alcohol, and neither the cells nor their vacuoles could be stained by oil red O, Sudan IV, or Lugol's iodine stains. Post-mortem smears were made of scrapings from the renal pelves of 17 fatalities. The large granular cell was present in every instance where death had occurred during the first two weeks. Smears of scrapings from the renal medulla revealed a small, degenerated, granular cell with similar characteristics.

Renal involvement resulted in azotemia, and studies were made of blood levels of uric acid (whole blood), urea nitrogen, and creatinine (Table I). The whole blood uric acid content was 5.2 to 12.0 mg. per 100 ml. in 16 of a series of 20 patients. The average duration of this increase was four days (range of 1 to 12 days), and maximum levels occurred between the fourth and twelfth days of illness. A value of 18.0 mg. per 100 ml. was noted on the twelfth day of illness in the one fatality in the series of 20. Blood urea nitrogen levels of 40 to 314 mg. per 100 ml. occurred in 59 of 76 patients, and values of 100 to 314 mg. per 100 ml. were present in 28 patients. The blood urea nitrogen began to increase between the second and fifth days of illness in 98 per cent of the 59 patients. Maximum levels occurred between the third and tenth days in 93 per cent, and abnormally increased values persisted for an average of eight days (range of 1 to 16 days). Blood creatinine contents were 2.5 to 20 mg. per 100 ml. in 58 of the 76 patients, 14 having values of 10 to 20 mg. per 100 ml. The blood creatinine began to increase between the second and sixth days of illness in 96 per cent of the 58 patients and reached a peak between the third and tenth days in 92 per cent. The average duration of creatinine elevation was eight days (range of 1 to 15 days).

TABLE I. BLOOD CHEMISTRY AND LIVER FUNCTION DATA IN PATIENTS WITH EPIDEMIC HEMORRHAGIC FEVER

LABORATORY DETERMINATION	NUMBER OF PATIENTS STUDIED	PATIENTS WITH ABNORMAL FINDINGS (PER CENT)	RANGE OF ABNORMALITY	DURATION (DAYS)		MAXIMUM ABNORMALITY (DAYS OF ILLNESS)
				AVERAGE	RANGE	
Blood uric acid	20	85	5.2-18.0 mg./100 ml.	4	1-12	4th-12th
Blood urea nitrogen	76	78	40-314 mg./100 ml.	8	1-16	3rd-10th
Blood creatinine	76	76	2.5-20.0 mg./100 ml.	8	1-15	3rd-10th
Blood sugar	10	0	*	-	-	-
Cephalin-cholesterol flocculation	13	85	++ to +++	10	7-16	6th-11th
Thymol turbidity	13	77	5-20 units	9	5-15	8th-12th
Serum bilirubin	13	23	1.2-2.0 mg./100 ml.	*	*	*
Serum alkaline phosphatase	10	20	5.7-6.7 units	4	3-4	6th-11th
Urine bilirubin	13	0	†	-	-	-
Urine urobilinogen	13	0	†	-	-	-

*This data was not available.
†No abnormality of this laboratory determination was observed.

Increases in blood hemoglobin content and hematocrit were noted for the second time after diuresis had started. Hemoglobin values of 16.0 to 20.3 Gm. per 100 ml. were noted at some time between the ninth and twentieth days in 38 patients (98 per cent of these between the ninth and fifteenth days). Hematocrit values of 48 to 61 per cent occurred at the same time in 48 patients.

4. *Hepatic involvement:* There were infrequent instances of hepatomegaly among the patients studied, and there have been pathologic reports of hepatic focal necrosis and mononuclear cell infiltration[1, 21, 22]; thus certain studies of liver function were performed (Table I).

Cephalin-cholesterol flocculation test results were 2 to 3 plus in 11 of a group of 13 patients for an average of 10-days (range of 7 to 16 days). The maximum cephalin-cholesterol flocculation reaction occurred between the sixth and eleventh days of illness. In 10 of these 13 patients, increased thymol turbidity levels of 5 to 20 units were present for an average of 9 days (range of 5 to 15 days). The maximum elevations occurred between the fifth and twelfth days of illness, 90 per cent between the eighth and twelfth days. The only patient in this series who died had an elevated thymol turbidity level for 15 days with a peak value of 20 units.

In general, there was a direct correlation between the thymol turbidity and cephalin-cholesterol flocculation values. The three patients with the highest thymol turbidity values, 15 to 20 units, had serum bilirubin contents of 1.2 to 2.0 mg. per 100 ml., while the serum bilirubin contents in the remaining patients were less than 1.0 mg. per 100 ml. Urine bilirubin analyses were negative, and urine urobilinogen determinations were positive in 1:10 dilutions and negative in 1:20 dilutions.

In 110 determinations of alkaline phosphatase in 10 patients, increased levels of 5.7 to 6.7 units were noted in two patients. The increased level persisted for three days in one of these patients and for four days in the other. The remaining alkaline phosphatase activity determinations were each less than 5 units.

In 10 patients, 115 blood sugar determinations were normal during a 14-day period.

DATA IN 22 FATALITIES*

In 22 persons who died, the maximum peripheral white blood cell counts were between 15,000 and 102,000 cells per cubic millimeter (over 20,000 cells in three-fourths of those who died) and occurred between the second and eighth days of illness. Data during the first few days of illness indicated a 50 per cent occurrence of transient leukopenia of 2,500 to 5,000 cells per cubic millimeter.

Adequate data concerning differential white blood cell counts were available in the records of 16 of the 22 fatalities. Myelocytes were present in 15 of the 16 for an average duration of seven days, reaching peak levels of 4 to

*A comparison between data in fatalities and data in those who survived is included under *Discussion.* (See also Table II.)

20 per cent in 14 of the 15. Metamyelocytes were noted in the blood smears of all 16 and attained peak levels of 5 to 18 per cent. Lymphoid cells described by Downey were also present in blood smears of all 16. The maximum percentage of Downey-type cells was less than 25 per cent in 15 patients and was observed subsequent to the sixth day of illness in 12.

From 3 to 15 blood platelet counts were performed in each of 15 patients who died. In 14 patients the lowest counts occurred between the third and ninth days of illness. These counts ranged from 30,000 to 130,000 platelets per cubic millimeter, and half of the 14 had counts of less than 75,000 platelets. Seven patients who survived the hemorrhagic phase had normal platelet counts between the sixth and seventeenth days of illness.

There were 3 to 15 absolute eosinophil counts recorded in each of nine fatalities. Each had at least one count over 300 cells per cubic millimeter, and maximum counts were between 1,100 and 4,400 cells in five. The highest counts occurred between the fifth and eleventh days of illness, and in five individuals they were noted immediately prior to death.

Each of 20 patients who died had 3 to 16 hemoglobin and hematocrit determinations during his illness. The range of maximum hemoglobin contents was 16.0 to 22.5 Gm. per 100 ml., and of hematocrit values, 50 to 64 per cent, and such increased levels occurred between the third and eighth days of illness. Hemoglobin contents of 18 to 22.5 Gm. per 100 ml. and hematocrit values of 55 to 64 per cent were recorded in 13 of the 20 individuals. Secondary increases in hemoglobin content (16.6 to 19.6 Gm. per 100 ml.) and hematocrit values (49 to 55 per cent) were noted in the four fatalities with the longest course of illness. Changes in the erythrocyte sedimentation rates in patients who died were of the same general pattern as in surviving patients.

Bone marrow specimens were obtained at the autopsies of 21 patients who died between the fourth and twenty-second days of illness. Myeloid: erythroid ratios ranging from 0.7:1 to 1.5:1 occurred in 12 marrow smears, while the rest had ratios from 1.6:1 to 2.9:1. Plasma cells comprised 2.0 to 5.7 per cent of the cells in nine marrow smears, and the atypical lymphocyte percentage varied from 1 to 6 per cent in ten marrow smears. Megakaryocytosis of 2.0 per cent was present in one specimen.

Daily measurements of urine volume were made in 19 patients who died. Volumes of less than 500 ml. per day were recorded in each of the 19, and volumes of less than 50 ml. were recorded in each of 6. This oliguria started between the third and eighth days of illness and lasted for 2 to 8 days. The average duration of oliguria could not be determined in patients who died since many were oliguric at the time of death, but the average duration was noted to be longer than 4.3 days. Diuresis with daily urine volumes of 2,200 to 6,200 ml. was observed between the twelfth and eighteenth days of illness in four patients. All patients who died had 4 plus proteinuria during the course of their illness. As far as could be determined, the pattern of change in urine specific gravity in surviving patients and in those who died was similar.

Of the 22 fatalities, 16 had from 3 to 16 determinations of blood urea nitrogen and creatinine levels prior to death. Maximum values for the former were 44 to 314 mg. per 100 ml. and for the latter were 2.5 to 26 mg. per 100 ml. These peak levels occurred between the sixth and twelfth days of illness, and in nine instances within 24 hours of death. As indicated in the previous section describing data in the group of 76 patients, there was only one death in each of the small series of patients in whom blood uric acid and liver function tests were studied. It was noted, however, that the person who died in the uric acid series had the highest blood uric acid content (18 mg. per 100 ml.), and the one who died in the liver function series had the highest thymol turbidity level (20 units).

DISCUSSION

To date, a specific cure for epidemic hemorrhagic fever has not been discovered, and the case fatality rate has been between 4 and 5.5 per cent at best.[1, 19, 23, 24, 25] Difficulties in arriving at an accurate diagnosis early in the course of the disease and the variable nature of the disease have hindered the setting up of therapeutic studies and have obscured the interpretation of the results of the studies undertaken. The importance of blood and urine findings in establishing a diagnosis has been indicated previously,[1, 19] and the laboratory data presented in this paper augment the quantitative information that is available concerning epidemic hemorrhagic fever. The discussion that follows indicates certain relationships of practical and theoretical importance.

In the 22 fatalities the general pattern of changes in the results of laboratory tests was similar to the pattern in patients who survived although differerences were noted in the degree, frequency, duration, and time of occurrence of these changes.

In Table II, differences in peripheral blood findings are indicated between the group of 76 patients with a 92 per cent case survival rate and the group with no survivals.

TABLE II. DIFFERENCES IN PERIPHERAL BLOOD FINDINGS IN EPIDEMIC HEMORRHAGIC FEVER BETWEEN 76 PATIENTS WITH A 92 PER CENT CASE SURVIVAL RATE AND 16 PATIENTS WITH NO SURVIVALS

	GROUP WITH 92 PER CENT SURVIVALS (% OCCURENCE)	GROUP WITH NO SURVIVALS (% OCCURENCE)
Maximum leukocytosis greater than 20,000 cells per cu. mm.	30	75*
Maximum myelocyte percentage greater than 4 per cent	25	88
Occurrence of atypical lymphoid percentage greater than 25 per cent	49	6
Occurrence of the maximum lymphoid percentage after sixth day of illness	11	75
Platelet count of less than 150,000 per cu. mm.	61	93†
Occurrence of at least one abnormal absolute eosinophil count	0‡	100§

*This is based on data in 22 patients.
†This is based on data in 15 patients.
‡This is based on data in 40 patients.
§This is based on data in 9 patients.

A maximum leukocytosis greater than 20,000 cells per cubic millimeter of peripheral blood was present in three-fourths of the patients who died, and in less than one-third of those who survived. In 88 per cent of the 16 fatalities with adequate data concerning differential blood cell findings, the maximum percentages of myelocytes were between 4 and 20 per cent, while one-fourth of those who lived had peak myelocyte levels greater than 2.8 per cent. The average interval during which myelocytes were present was 7 days in fatalities and less than 4 days in patients who survived. Peak myelocyte levels were not related to the time of death, and ranges of metamyelocyte and stab differential counts were similar in those who died and those who lived. In 34 (49 per cent) of the 70 patients who survived, the level of atypical lymphoid cells exceeded 25 per cent at some time, while this level was exceeded in only one of the 16 fatalities. In 89 per cent of patients who survived the disease, the maximum number of lymphoid cells was observed in blood smears before the sixth day of disease, while in 75 per cent of the fatalities, the maximum number of these cells was not observed until after the sixth day.

Peripheral blood platelet counts of less than 130,000 per cubic millimeter were recorded in 93 per cent of the fatalities, while counts of less than 150,000 platelets per cubic millimeter were observed in less than two-thirds of those who survived. At least one abnormally increased absolute eosinophil count was recorded in each of the nine fatalities in whom such counts were performed, whereas these counts were within normal limits in surviving patients.

A decreased myeloid:erythroid ratio was apparent in the bone marrows of all patients who died. In addition, there was an increase in the numbers of plasma cells and atypical lymphocytes in about one-half of the marrow specimens. In the examination of marrow specimens from 11 patients who survived, it was noted that 6 had decreased myeloid:erythroid ratios.

The pattern of hemoconcentration as evidenced by increases in blood hemoglobin and hematocrit was similar in surviving and non-surviving patients. These increases tended to be greater in the latter group in which 65 per cent had blood hemoglobin contents of 18 to 22.5 Gm. per 100 ml. and hematocrit values of 55 to 64 per cent.

Nonsurviving patients differed from surviving ones in that 100 per cent of the former had proteinuria of 4 plus degree, and 84 per cent of the latter had 3 plus to 4 plus proteinuria. In addition, each of those who subsequently died had oliguria for an average duration of more than 4 days, while two-thirds of those who lived had oliguria which had a shorter average duration of 2 days. Similarly, abnormally increased levels of blood urea nitrogen and blood creatinine occurred more commonly in the fatal group. In the small groups of patients in whom a study was made of blood uric acid values and thymol turbidity results, only one patient died in each group, and it was noted that the greatest abnormality in each case occurred in the one who died.

On the basis of the data that have been presented, it is possible to indicate the following features that relate to diagnosis, to prognosis, and to therapeutic studies:

1. In hospitalized patients with epidemic hemorrhagic fever, certain abnormalities were characteristically evident. The presence of most of these ab-

normalities was required in order to establish the diagnosis with any degree of certainty, since there was no specific diagnostic test for the disease. Immature cells of the myeloid series apeared in the peripheral blood during the first few days of illness as part of an increasing leukocytosis. This so-called "leukemoid response" was replaced by a lymphocytic response toward the end of the first week, and many atypical lymphoid cells were noted. During the first week of illness, the peripheral blood platelet count was decreased abnormally, and nucleated red blood cells and macroplatelets were observed in differential blood smears. The onset of proteinuria and oliguria was noted, and the urine contained an increased number of red blood cells, granular cells (as previously described in this report), and casts. Urine specific gravity decreased. Hemoconcentration was evident, and the erythrocyte sedimentation rate increased. At this time and extending into the second week, blood chemical changes included increased urea nitrogen, creatinine, and uric acid levels with occasional alterations in the results of liver function tests. Associated with the onset of diuresis, there was a second episode of hemoconcentration, and abnormal changes returned to normal. The peripheral white blood cell count, erythrocyte sedimentation rate, and renal concentrating function were usually last to return to normal.

2. Laboratory findings that aided in establishing the early diagnosis of epidemic hemorrhagic fever included the presence of myelocytes in peripheral blood, increased numbers of red blood cells in urine, and protein in urine. It remained true, however, that blood and urine findings were frequently normal during the first three days of illness, and one could do generally no more than suspect the presence of this disease in persons who were exposed in endemic areas and who subsequently became acutely ill with a febrile illness of unknown etiology.

3. No absolute criteria for predicting severe disease were discovered; nevertheless, a prognosis of severe disease was indicated generally by such findings as peripheral white blood cell count greater than 20,000 cells per cubic millimeter, peripheral blood myelocyte percentage greater than 4 per cent, duration of leukemoid response averaging more than 7 days, delayed appearance of the maximum number of atypical lymphoid cells beyond the sixth day of illness, peripheral blood platelet count of less than 100,000 cells per cubic millimeter, abnormally increased peripheral blood eosinophil count, marked hemoconcentration (hemoglobin levels greater than 18 Gm. per 100 ml. and hematocrit values greater than 55 per cent), oliguria averaging 4 days or more, and marked increase in the blood levels of urea nitrogen, creatinine, and uric acid.

4. In defining the laboratory diagnostic criteria that are required for including a patient with early illness in a therapeutic study (controlled experiment to test the efficacy of a therapeutic agent), the leukemoid response with its increased numbers of myelocytes and metamyelocytes is useful. The presence of increased numbers of red cells in the urine or proteinuria are helpful only if present. By the third to fifth days, all three abnormalities as well as findings of thrombocytopenia and oliguria will have become impor-

tant as criteria for the inclusion of patients in therapeutic studies. A group of laboratory criteria can be defined for the study of therapeutic agents in severe illness by including one or more of the laboratory findings that were listed in paragraph 3 of this section as being generally prognostic of severe disease. Although the manifestations and course of disease are variable, controlled therapeutic studies based on such laboratory criteria and based on similarly well-defined clinical criteria would permit the use of smaller numbers of patients than heretofore possible.

5. In future studies, objective laboratory evidence relating to the efficacy of therapeutic agents can be obtained by an evaluation of the degree and duration of leukocytosis, thrombocytopenia, proteinuria, oliguria, and azotemia. Similar clinical evidence can be obtained from an evaluation of the total duration of illness and case fatality rate, as well as from evaluations of the degree and duration of a few representative features of the febrile, hemorrhagic, hypotensive, oliguric, and convalescent phases.

SUMMARY

Hematologic, urine, and blood chemistry findings were assembled from 92 United Nations patients who had epidemic hemorrhagic fever during an outbreak of the disease between April and July, 1952. Leukocytosis and a leukemoid response occurred in the peripheral blood during the acute febrile phase, and, soon thereafter, a lymphocytic response with the appearance of many atypical lymphoid cells was noted. A decreased number of peripheral blood platelets was observed during the hemorrhagic phase. Proteinuria, microscopic hematuria, granular celluria, oliguria, and azotemia appeared during the first week of illness, and renal concentrating function decreased. During the hypotensive or shock phase hemoconcentration was evidenced by abnormally increased blood hemoglobin and hematocrit values. The onset of diuresis during the second week of illness was usually associated with the onset of convalescence, during which abnormal findings returned to normal. In general, the white blood cell count, erythrocyte sedimentation rate, and renal concentrating function were last to return to normal.

Abnormal laboratory findings in patients who subsequently died were of the same type as findings in patients who survived. Abnormalities of greater severity and more prolonged duration, however, occurred more commonly in fatal cases.

Problems in diagnosis and prognosis are discussed, especially with regard to the early phase of the disease. On the basis of this discussion and on the basis of the data presented, laboratory criteria are proposed for the inclusion of patients in controlled studies to test the efficacy of therapeutic agents, and laboratory criteria are proposed for the evaluation of such studies.

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THE SIMILARITY OF BACTERICIDAL RATES OF PENICILLIN FOR STREPTOCOCCUS VIRIDANS FROM SUCCESSFULLY TREATED CASES OF BACTERIAL ENDOCARDITIS AND FROM CASES WHICH RELAPSED

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WHEN subacute bacterial endocarditis caused by strains of streptococcus viridans sensitive to penicillin is treated with this antibiotic, relapse rates of 10 to 25 per cent have been frequently observed. This seems to be the case when patients are treated with large doses of penicillin for short periods[1] or with smaller doses for longer periods.[2]

The reasons for these relapses remain a matter of speculation. An increase in resistance of the streptococci to penicillin has not been observed. Streptococci from patients who have relapsed as many as three and four times have remained as sensitive as they were prior to treatment. The possibility that penicillin may penetrate better into the depths of certain vegetations than others is a real one, but one which as yet has been neither proved nor disproved. Another possibility is that though the "penicillin sensitivity," as measured by the ordinary serial dilution test in broth, is the same for strains recovered from successfully treated patients as from those who relapsed, the streptococci may be killed more rapidly in the successfully treated patients.

In order to investigate this possibility, penicillin bactericidal growth curves have been determined for 5 strains from successfully treated patients and for 5 strains from 3 patients who relapsed. Of the latter, 2 were recovered prior to treatment and 3 during a relapse. It is the purpose of this communication to report these studies.

METHODS

Prior to the study, the streptococci were kept either in lyophilized form or frozen and stored at -15° C. At the time of study they were cultured in brain-heart infusion broth. Sixteen- to twenty-hour cultures were used as inocula for the bactericidal tests. The latter were performed in Erlenmeyer flasks containing 45 ml. of brain-heart infusion broth, 3 ml. of Kahn-positive human bank blood, 1 ml. of the appropriate penicillin solution, and 1 ml. of the bacterial inoculum, making a total of 50 ml. The penicillin was crystalline penicillin G potassium dissolved in sterile distilled water.

The flasks were incubated at 37° C., until subcultures were negative for 2 successive days. At the end of each 24 hours, 1.2 to 2.2 ml. was removed for subculture in broth and in

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agar. Quantitative bacterial counts were made from pour plates prepared either from the undiluted broth removed from the flasks or from an appropriate dilution. In preparing the subcultures 0.2 ml. of penicillinase was added to each culture tube or pour plate.

RESULTS

Penicillin Treatment Received by the Patients.—Table I summarizes the treatment of the patients in this series. The 5 successfully treated patients received either 15 million or 16 million units of crystalline penicillin G daily for 14 or 15 days.[1] All are alive and most are leading normal lives and working or attending school 2½ to 5½ years after the completion of therapy.

TABLE I. PENICILLIN TREATMENT RECEIVED BY PATIENTS

Successfully Treated Patients			
PATIENT	DAILY DOSE OF PENICILLIN (UNITS)	NO. OF DAYS	DURATION OF FOLLOW-UP (YEARS)
H. N.	16,000,000	14	5
W. P.	16,000,000	14	5½
M. W.	15,000,000	14	4½
A. T.	15,000,000	15	2½
M. F.	15,000,000	14	2½

Patients Who Relapsed										
				CTC*		CAMP†		SMC‡		
PATIENT	COURSE	DAILY DOSE OF PENICILLIN (UNITS)	NO. OF DAYS	GM./ DAY	DAYS	GM./ DAY	DAYS	GM./ DAY	DAYS	CLINICAL RESULT
L. B.	1	10,000,000	13							Well 2½ yr.
	2	15,000,000	21	2	4	2-4	25			
	3	20,000,000	15							
	4	15,000,000	29					2	29	
	5	81,000,000	25							
Aq.	1	6,000,000	14					2	14	Well 1½ yr.
	2	6,000,000	8					2	15	
		3,000,000	7							
	3	1,600,000	42							
Rish.	1	6,000,000	14					2	14	Did not return

*Chlortetracycline.
†Chloramphenicol.
‡Streptomycin.

Patient L. B. relapsed 4 times following large doses of penicillin or penicillin plus chloramphenicol, chlortetracycline, or streptomycin. She was finally cured after receiving 81 million units of penicillin daily for 25 days, and has been leading a normal life for more than 2½ years. Patient Aq. relapsed twice after courses of 6 million units of penicillin plus 2 Gm. of streptomycin daily for 14 and 15 days each, a regimen similar to those used successfully by Hunter[3] and by Geraci and Martin.[4] She has been well for more than one year following a six weeks' course of 1.6 million units daily. Patient Rish. relapsed after a single course of 6 million units of penicillin and 2 Gm. of streptomycin daily for 14 days, left the hospital and did not return.*

*Patients Aq. and Rish. were treated at the New York Hospital. Their case histories and the strains of *Streptococcus viridans* recovered from their blood streams were kindly sent to us by by Dr. Ralph Tompsett. L. B. was treated at C. Hospital and later at the Cincinnati General Hospital. The other patients were treated in the Cincinnati General Hospital.

Minimal Inhibiting Concentration of Penicillin for the Strains Investigated.—Table II presents the minimal inhibiting concentration (M.I.C.) of penicillin, as determined by an overnight twofold serial dilution test,[5] for the streptococci studied. The M.I.C. for the successfully treated patients varied from 0.025 to 0.1 μg per milliliter.

TABLE II. MINIMAL INHIBITING CONCENTRATION (M.I.C.) OF PENICILLIN FOR STRAINS INVESTIGATED

STRAIN	SPECIES	M. I. C. (μG/ML.)
	Successfully Treated Patients	
H. N.	*Str. salivarius**	0.025
W. P.	*Str. thermophilus**	0.05
M. W.	*Str. salivarius*	0.1
A. T.	*Str. viridans*, not further identified	0.05
M. F.	*Str. salivarius*	0.1
	Patients Who Relapsed	
L. B.	*Str. sanguis*	0.012
Aq. I	*Str. thermophilus*	0.025
Aq. II	*Str. thermophilus*	0.025
Rish. I	*St. acidominimus*	0.025
Rish. II	*Str. acidominimus*	0.025

*Probable identification.

The L.B. strain, recovered after her fourth relapse, was inhibited by 0.012 μg per milliliter. The strain designated Aq. I, the pretreatment organism, was inhibited by 0.025 μg per milliliter, as was Aq. II, the strain recovered after the first relapse. Both Rish. I, the pretreatment strain, and Rish. II, cultured during the relapse, required 0.025 μg per milliliter.

TABLE III. NUMBER OF DAYS REQUIRED FOR PENICILLIN TO STERILIZE BROTH CULTURES

BACTERIAL INOCULUM	PENICILLIN, μG PER ML.		
	50	2	0.5
	"Relapse" strains		
Undiluted culture	6.6	7.0	6.5
10^{-3}	3.4	3.8	3.6
10^{-6}	1.4	1.6	1.4
	"Nonrelapse" strains		
Undiluted culture	7.0	8.5	7.0
10^{-3}	5.1	4.5	3.3
10^{-6}	2.2	2.0	1.8

Each figure is the average of several experiments performed with each strain in the 2 groups.

Time Required for Penicillin to Sterilize Cultures.—The data in Table III show that the number of days required for penicillin to sterilize cultures from patients who relapsed was essentially the same as for patients who were successfully treated. Furthermore, the number of days required for sterilization did not differ for the 3 concentrations of penicillin studied, 50, 2, and 0.5 μg per milliliter. The important variable proved to be the size of the bacterial inoculum. In both groups of streptococci, and at all concentrations of penicillin, 5 to 6 days longer were required to sterilize an inoculum of undiluted culture than 10^{-6} dilution.

Earlier investigators[6-8] have called attention to the *rate* of bactericidal action of penicillin and have at the same time emphasized the time required to kill 99.9 per cent of the inoculated bacteria. In each of our experiments, regardless of the origin of the strain, concentration of penicillin, or size of bacterial inoculum, 99.9 per cent of the bacterial cells were killed by the end of 24 hours. This point is best brought out by inspection of the slopes of the curves shown in Figs. 1 and 2, prepared from one "relapse" strain (L. B.) and one "nonrelapse" strain (W. P.).

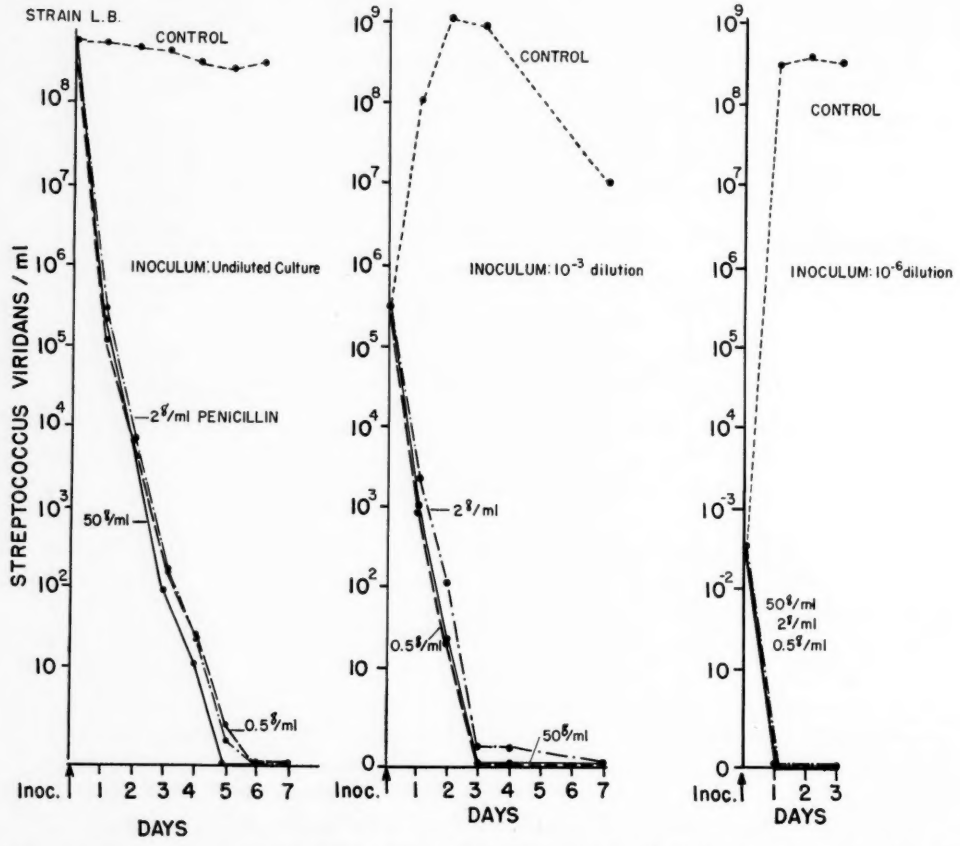

Fig. 1.—Rate of bactericidal action against *Streptococcus viridans* from a case of subacute bacterial endocarditis which relapsed following treatment.

A Strain Requiring Longer for Complete Sterilization.—One of the 10 strains, H. N., from a case successfully treated by a single two-week course of penicillin, required at least half again as long for complete sterilization as did any of the other 9. The growth curves on this strain were repeated many times, sometimes with different inocula on different days, but sometimes with all 3 inocula at all 3 penicillin concentrations simultaneously. Such an experiment provided the data for Fig. 3.

DISCUSSION

It is evident from these experiments that the rate of bactericidal action and the period of time required to complete sterilization of broth cultures are the same for streptococci recovered from successfully treated patients as for patients who relapsed. In a disease like bacterial endocarditis, where the therapeutic result depends entirely upon chemotherapy, complete eradication of the infecting bacteria is probably necessary. In order to accomplish this, a bactericidal concentration of penicillin may need to be maintained within the vegetation until the last streptococcus is dead. Increasing this concentration above the minimal bactericidal level will probably not be therapeutically useful, but

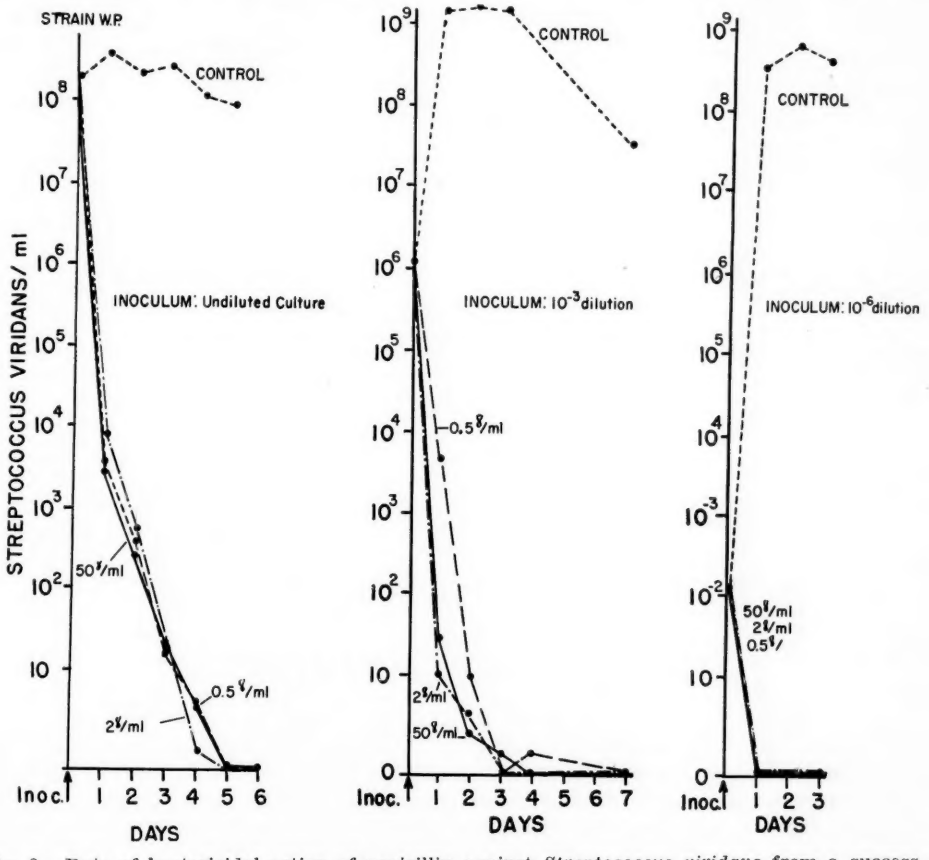

Fig. 2.—Rate of bactericidal action of penicillin against *Streptococcus viridans* from a successfully treated case of subacute bacterial endocarditis.

allowing it to fall below this level could lead to failure. We may assume that the relapses in the 3 patients reported in this paper were not due to any characteristic of the streptococci themselves, but rather to a failure to achieve and to maintain an adequate bactericidal concentration of penicillin within the vegetation. It would follow, therefore, that the blood level of penicillin in any given patient with bacterial endocarditis is important only in so far as it re-

flects the level of penicillin within the vegetation, a variable which we cannot measure clinically. In the 5 successfully treated patients there was evidently enough penicillin continuously at the site of the lesion to effect cure.

It is well known that a few patients have been adequately treated with doses as low as 300,000 units of penicillin daily for three weeks. The disparity in minimum daily dose and minimal duration of therapy required for patients in general suggests that variable numbers of streptococci are present in different vegetations and that the penetrability of vegetations by penicillin is not uniform.

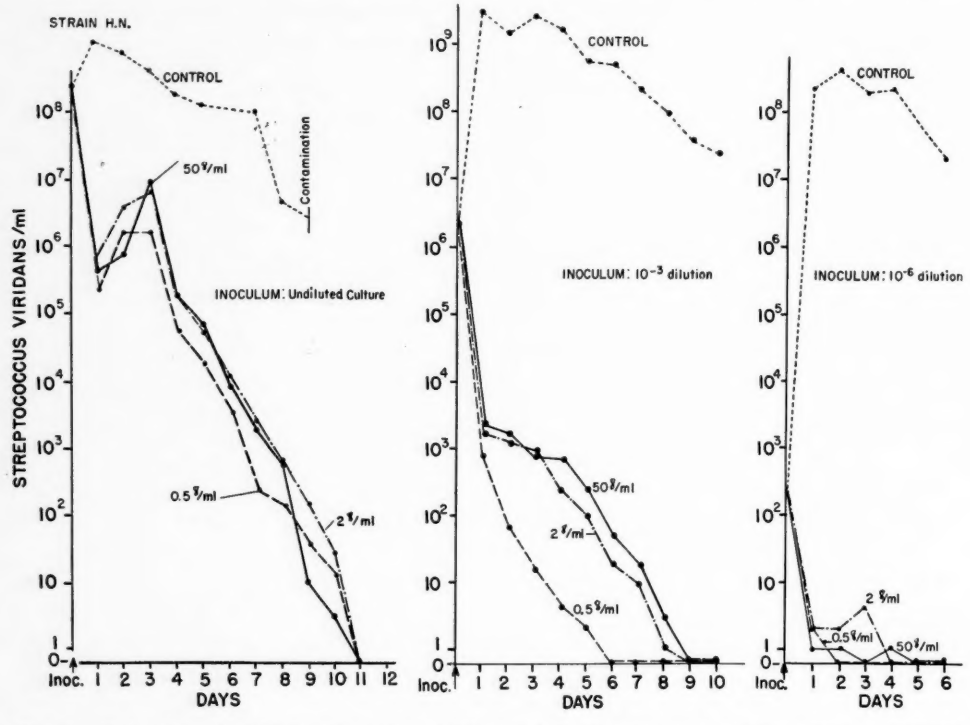

Fig. 3.—Rate of bactericidal action of penicillin against *Streptococcus viridans* from a successfully treated case of subacute bacterial endocarditis.

SUMMARY

The rate of bactericidal action of penicillin and the number of days required to sterilize cultures were determined in a series of 5 strains of penicillin-sensitive *Streptococcus viridans* recovered from successfully treated patients with bacterial endocarditis, and 5 strains from 3 patients who relapsed following similar treatment. The rate of bactericidal action and the time required to sterilize broth cultures were practically the same for the strains from both groups of patients. Furthermore, the effects were practically identical when 3 different concentrations of penicillin, 0.5, 2, and 50 μg per milliliter were investigated.

The number of bacteria inoculated proved to be an important variable. Small bacterial inocula, that is 10^{-6}, required on an average of 1.4 to 2.2 days

for complete sterilization. Medium-sized inocula, that is 10^{-3}, required 3.3 to 5.1 days for complete sterilization, whereas undiluted culture required 7 to 8.5 days. Thus, the density of bacterial population was more important in determining the sterilization time of these cultures than the source of the culture or the concentration of penicillin, provided that the latter exceeded the minimal inhibiting concentration.

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THE REACTION OF SEROUS CAVITIES TO BLOOD

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THE existence, as well as the nature and the mechanism, of response of serous cavities to blood has never been fully studied. It was the purpose of this investigation to determine: (1) if there is a regular cellular response; (2) if such response exists, what may be its character.

From clinical reports[1-5] it is evident that uncontaminated intraperitoneal bleeding gives rise to peritoneal pain, fever, leukocytosis and, in some instances, shock. The protocols of the study of peritoneal pain carried out by Mengert, Cobb, and Brown[6] reveal that several of their patients who received intraperitoneal injections of their own blood developed within two to six hours a mild leukocytosis (12,000) and slight temperature elevation (100° F.).*

That such a reaction is not peculiar to the peritoneum is suggested by personal experience in injuries of the pleura which result in aseptic hemothorax. There is, as in hemoperitoneum, mild fever and leukocytosis. Studies of the aspirated fluid have revealed a cellular distribution different from that of the circulating blood. The relative proportion of cells also varies with the elapse of time following injury. Schwartz and Broadbent[8] have made similar observations.

Of particular interest is a group of patients in whom unusually high eosinophil counts were found in pleural fluid.[8-13] All writers are of the opinion that the duration of the fluid had to be fairly considerable, from 10 days upward. Although the nature of pleural injury varied greatly, from study of the protocols it is evident that if any single substance was present in and common to all these, it was the individual's own blood. Scott and Finland[13] were of the opinion that the presence of red blood cells had nothing to do with eosinophilia, but MacMurray and associates[12] can find no other common condition in the several cases of eosinophilic pleurisy they dealt with.

A review of the experimental literature indicates that, in the past, various studies in regard to the behavior of blood in these two major serous cavities have been carried out, but with many different purposes. No continuous study of the reaction of a serous cavity to blood has come to the writer's attention. Certain of the studies have related to the absorption of blood from the peritoneum, the rate, the channels through which it passes, the effect of respiration on ab-

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sorption, and the site of absorption.[14-21] Others have dealt with efforts to produce an eosinophilia of the cavities.[22-30] Yet others have studied the relative rates, sites, and routes of absorption of crystalloids, colloids, and red cells.[29]

There are scattered bits of information suggesting that blood in a serous cavity is not entirely innocuous and that it produces some type of cellular reaction. On the one hand are clinical cases with an early peripheral leukocytosis with increase in polymorphonuclears. On the other hand there are the reports of chronic pleuritis with varying degrees of eosinophilia. It seemed desirable to undertake a study in which the local and systemic reactions to intraperitoneal blood might be followed at regular intervals up to the time of their subsidence.

Moreover, if it should be shown that blood does indeed provoke a reaction, it might become possible to explain certain features about fluids which sometimes makes them very difficult to classify as either exudate or transudate. For, clinically, it is in sterile fluid with small amounts of red cells (a type commonly encountered in carcinomatous metastases, infarcts, and the like) that the fluid seems to fall into an intermediate type that is not clearly an exudate, yet which certainly cannot be termed a transudate.

MATERIALS AND METHODS

Pedigreed white mice of the same age were used throughout this series of experiments. Into each of them was injected on a single occasion 1 ml. of freshly drawn human blood which had been treated according to the methods described subsequently in each experiment. The material was injected into the peritoneal cavity after preparation of the abdomen by thorough washing and rinsing with 70 per cent alcohol. When clotted blood was the material chosen, an 18 gauge needle was used. Under other conditions of the blood a 21 or 22 gauge needle was chosen. All materials were handled with strict aseptic technique.

At the time chosen for sacrifice the animal was killed by a blow on the head. The abdomen was washed with 70 per cent alcohol and aseptically opened from symphysis to xiphoid. Swabs were made at once from the peritoneal surface and were streaked on slants for culture. An ordinary glass slide was then drawn across the peritoneal surface, fixed and stained with Wright's stain. Subsequently a differential count of from 200 to 500 cells was made. No effort was made to count the total number of cells in the exudate by the method of Panzenhagen and Spiers,[25] which had not appeared at the time this work was first undertaken. The concern, moreover, was rather with the character of the response than with the amount. In addition, it was feared that, if saline was used to wash off the expected exudate, handling might result in the disruption of some cells while others might adhere to the peritoneal surface.

White and differential counts of peripheral blood were made by snipping off a bit of the tail and carrying out the usual procedures. At the beginning of these experiments these counts were performed routinely, but it became apparent after a considerable number had been performed that they contributed no important finding, and they were accordingly discontinued.

RESULTS

Experiment 1.—Twelve white mice were injected intraperitoneally each with 1 ml. of human blood which had been treated in the following manner: The blood was withdrawn from the donor's vein, and introduced into a sterile test tube where it was allowed to stand until a soft clot had just formed. The clot was then broken to bits with sterile applicator sticks and the suspension drawn up through an 18 gauge needle into a syringe. Peripheral counts on two of the

TABLE I. REACTION OF MOUSE TO 1 ML. CLOTTED HUMAN BLOOD INJECTED INTRAPERITONEALLY

		PERIPHERAL BLOOD					PERITONEAL SMEARS*		
ANIMAL	TIME OF SACRIFICE	TOTAL W.B.C.	BAND (PER CENT)	SEGMENTED (PER CENT)	LYMPHOCYTE (PER CENT)	EOSINOPHIL (PER CENT)	POLYMORPHONUCLEAR (PER CENT)	LYMPHOCYTE (PER CENT)	EOSINOPHIL (PER CENT)
1	24 hour	10,700	7	28	46	19	74	21	5
2	48 hour†	18,450	3.5	19.5	74	3	20	72	8
3	48 hour†	8,675	2.5	22.5	71	3.5	70	24	6
4	72 hour	7,300	2.5	21	70	6.5	23	65	12
5	72 hour	8,750	.5	10.5	81.5	7.5	29	62	9
6	96 hour	9,100	3	29	59	9	20	69	11
7	96 hour	12,150	4	33.5	51	11.5	31	60	9
8	5 day	15,000	2	44	42	12	27	66	7
9	5 day	10,650	3.5	77.5	66	8	19	68	13
10	5 day	10,125	.5	22.5	65	11.5	40	50	10
11	5 day	24,870	1.5	20.5	75	2	(injured)		
12	7 day	11,100	1	35.5	58.5	5	14	79	7
13	9 day	7,900	0	19.5	77	3.5	13	74	13
14	9 day	8,400	2.5	24	50	23.5	24	65	11
15	11 day	12,900	6	38	53	4	10	24	66
16	11 day	5,150	1	10.5	60	28	31	35	34
17	13 day	12,950	1.5	15.5	74.5	8	19	63	18
18	13 day	3,350	8	26	55	11	6	77	16
19	13 day		11	19	65.5	4.5	4	83	10
20	15 day	12,650	4.5	45	45	5.5	27	55	18
21	15 day	8,200	7.5	28.5	59.5	8.5	1	92	7
22	17 day	8,100	5	26.5	53	15.5	6	56	38
23	17 day	1,900	2	23	48	27	1	87	9
24	21 day	13,850	2.5	18	57.5	22	2	89	8
25	21 day	12,350	3	25	60	12	8	88	3
4 control animals Average		9,000	25	42	54	1.5		99	1
6 control animals injected with saline—Average (5 days)								99	1

*Human polymorphonuclears excluded from count.
†No visible blood in peritoneal cavity. No human polymorphonuclears seen in peritoneal films, from this time on.

mice 10 minutes prior to injection revealed 3 and 4 per cent band forms, 42 and 36 per cent segmented forms, 50 and 55 per cent lymphocytes, and one had 10 per cent eosinophils.

These two animals were sacrificed 4 hours later, and at that time their peripheral differential counts were 23 and 26 per cent bands, 44 and 43 per cent segmented forms, and 33 and 28 per cent lymphs. The blood was found to be in a semiliquid state, pretty evenly distributed throughout the peritoneal surface, which showed a slight haziness. Differential counts of the peritoneal smears were difficult on account of the presence of so much human blood, but the mouse polymorphonuclear cell can be distinguished by its multilobulated state from human neutrophilic leukocytes. The distribution between human and mouse leukocytes appeared to be roughly equal, but it was impossible to separate lymphocytes from the two species.

Two more animals were sacrificed 24 hours later, but since injury to the bowel was present in one, it had to be discarded. Thereafter animals were sacrificed every 24 hours through 120 hours. The results of this experiment are summarized in Table I.

Since evidence of reaction in the peritoneum was present as late as five days, 16 more animals were treated in precisely the same fashion, but were sacrificed at the end of 5, 7, 9, 11, 13, 15, 17, and 21 days, both peripheral counts and smears from the peritoneum being made in all of these (Table I).

Control 1: Four normal healthy litter mates were sacrificed without having received any injection of any kind. Smears from their peritoneums revealed a very few scattered cells, which in 3 consisted entirely of lymphocytes and some very large cells which apparently were mesothelial in origin. One smear revealed 1 eosinophil. The range of peripheral counts on these animals was from 5,900 to 15,500. Bands averaged 2.5 per cent; segmented forms ranged more widely, 15.5 per cent to 55 per cent; and the highest peripheral eosinophil count was 3.5 per cent.

Control 2: Six healthy white mice were injected with 1 ml. normal saline and were sacrificed at the end of day 5. Smears from their peritoneal surfaces at that time revealed the same distribution of cells as those taken from untreated mice. The highest number of eosinophils encountered in any slide was 3, in 200 cells counted.

Within 4 hours after injection of blood there was a definite left shift evident in the peripheral blood and a polymorphonuclear reaction in the peritoneal film. At the end of 24 to 48 hours the peritoneum was entirely free of visible blood, though sometimes brownish discoloration of the underside of the diaphragm above the liver was visible, an observation supported by experiments of others which indicate the route of absorption of particulate matter is through diaphragmatic lymphatics.[15, 16] At 48 hours the peripheral count had returned to normal. In the peritoneal film were many lymphocytes and in addition there were numbers of large cells, apparently mesothelial in origin, plus fairly numerous large mononuclear phagocytic cells, which may well have had a local tissue origin (Fig. 1).

Fig. 1.

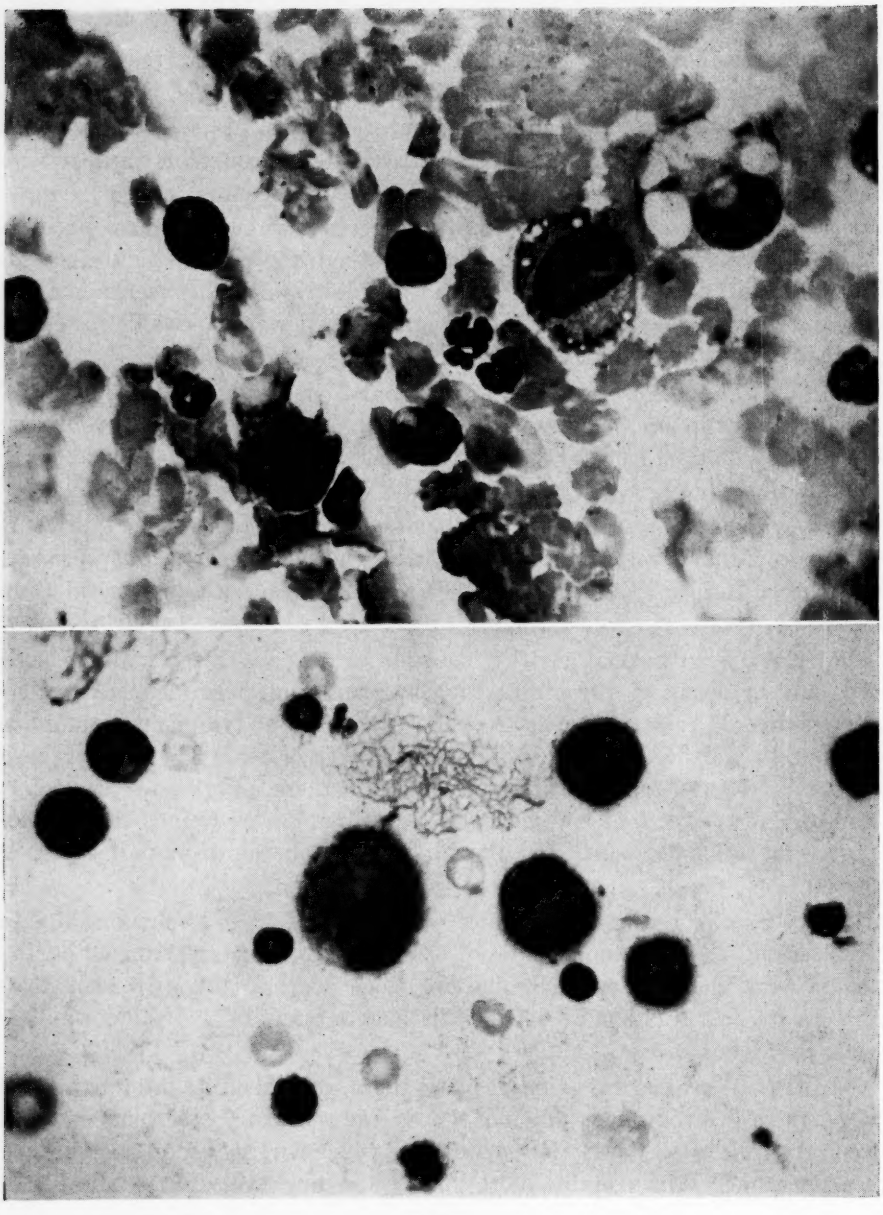

Fig. 2.

Fig. 1.—Characteristic peritoneal smear at end of 48 hours. Much residual blood is present. Three highly lobulated mouse polymorphonuclears are readily recognized. Note vacuolization of two upper cells at right.

Fig. 2.—Peritoneal smear at end of 72 hours. Injected blood is almost completely absent. The three largest cells in the center are probably of peritoneal origin. Two large mononuclear phagocytes lie to each side. The small cells are lymphocytes.

Fig. 3.

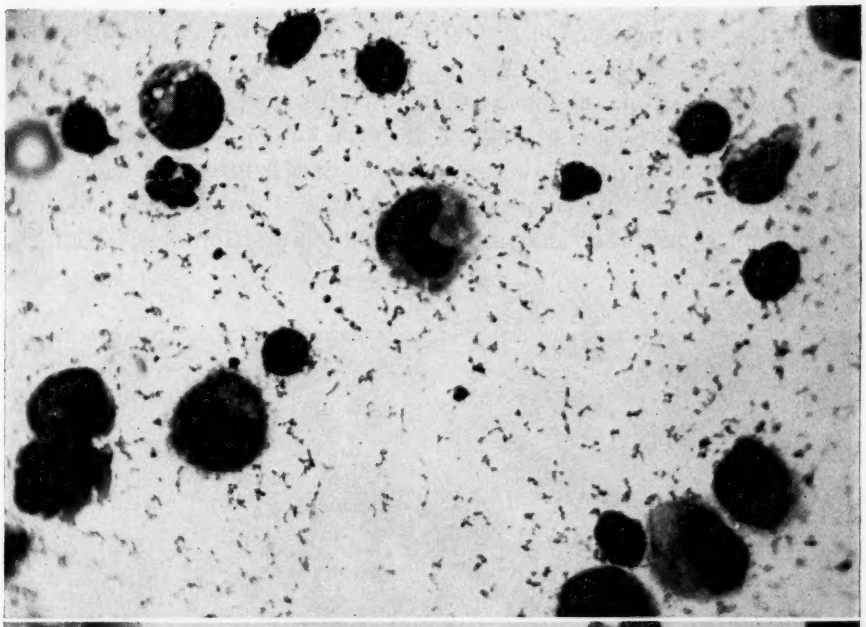

Fig. 4.

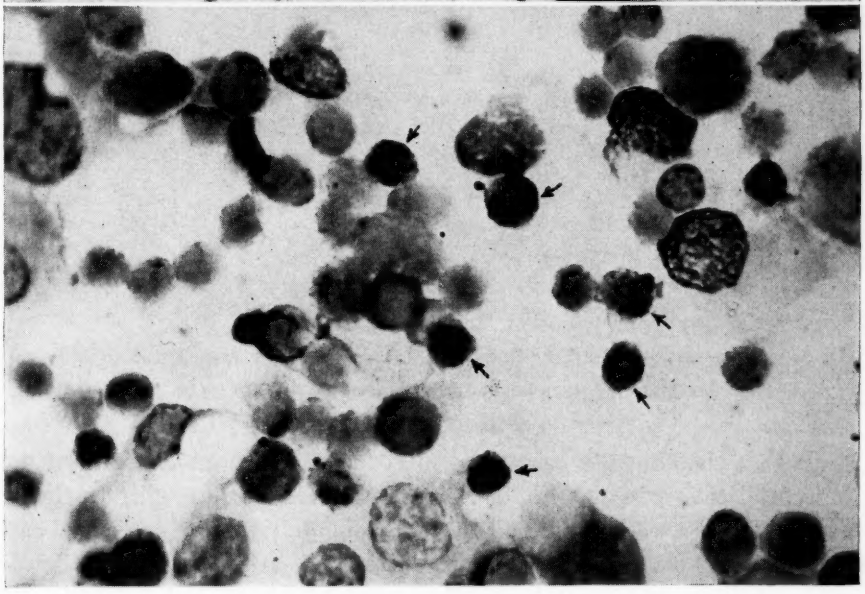

Fig. 3.—Peritoneal smear at end of 96 hours. A number of mononuclear phagocytes, one containing vacuoles is seen. Beneath this is the extensively lobulated nucleus of a mouse polymorphonuclear. Cells showing eosinophilic granulation are at 11 o'clock and at 3 o'clock.

Fig. 4.—Smear from mouse peritoneum at end of eleven days. Cells indicated show eosinophilic granulation. The numerous shadowy cells in the background take a light blue stain and may be degenerating cells of lymphocytic or mononuclear series or may represent nuclei of degenerating peritoneal surface cells.

At 72 hours a rising peripheral blood eosinophilia of 5 to 6 per cent was noted, and eosinophils were making their appearance in as high as 6 to 8 per cent of the peritoneal cells, the rest of which consisted of large mononuclears and lymphocytes (Fig. 2).

At 96 hours, peripheral blood eosinophilia reached an average of 10 per cent and the numbers of eosinophils in the peritoneal slides ran as high as 20 to 30 per cent, with the distribution of other cells as before (Fig. 3).

By 120 hours after injection of blood, peripheral eosinophilia had declined slightly but not significantly and the peritoneal smears contained about 25 per cent eosinophils.

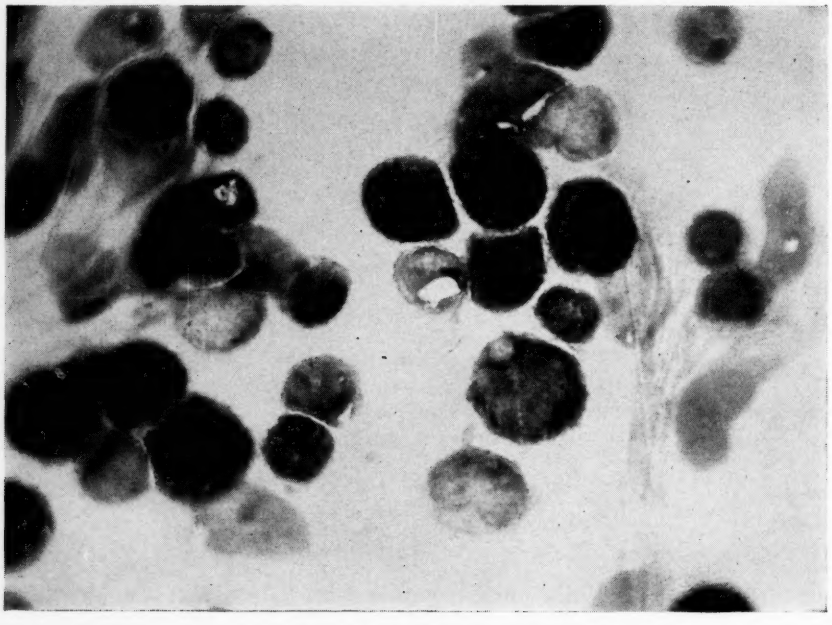

Fig. 5.—Peritoneal smear at the end of 72 hours. Stained with Prussian blue reagent and with safranin. The large cell at the lower center contains much Prussian blue staining material. Smaller deposits of the same material are seen as black dots in some of the cells around the periphery.

From that time on, the peripheral total leukocyte counts were normal and the differential counts in about half the animals were also normal. In the other half there was still a well-marked peripheral eosinophilia. The peritoneal smears from 7 to 21 days showed very few polys (range 1 to 10), many lymphocytes, scattered monocytes, and an average of 24 per cent eosinophils, the highest percentage being 66 and the lowest (one animal at the end of 21 days) 3 (Fig. 4).

Tissues of these mice, very kindly examined by Dr. A. J. Gill, revealed nothing very striking. In fixed tissue studies evidence of peritonitis was wholly lacking. Minor degrees of reactive hyperplasia were noted in the spleen, and fairly noticeable amounts of hemosiderin were observed in the livers and spleens of animals sacrificed in the 3- to 5-day period.

In not one of these animals was there noted any evidence of peritonitis nor were their peritoneal adhesions in mice sacrificed after the longer intervals.

In peritoneal slides made 72 hours after injection and longer, it was noted that the large mononuclears contained numerous yellow-brown granules. Inside a few of these phagocytes almost intact red cells could be seen. On staining with the Prussian blue reagent, it was seen that these granules were composed of iron-containing pigment (Fig. 5).

TABLE II. REACTION OF RATS TO AUTOGENOUS BLOOD 5 DAYS AFTER INJECTION OF 1 ML. CARDIAC BLOOD INTO PERITONEAL CAVITY

		POLYMORPHONUCLEAR (PER CENT)	LYMPHOCYTE (PER CENT)	MONONUCLEAR* (PER CENT)	EOSINOPHIL (PER CENT)
R1	Peripheral Blood	6	93		1
	Peritoneal Smear		62		38
	Pericardial Smear	0.5	79		20.5
R2	Peripheral Blood	2	96	1	1
	Peritoneal Smear	2	78		20
	Pericardial Smear	1	86.5		13.5
R3	Peripheral Blood	9	90		1
	Peritoneal Smear		80		20
	Pericardial Smear		76		24
R4	Peripheral Blood	12	79	2	7
	Peritoneal Smear	2	64		34
	Pericardial Smear	5	94		1
R5	Peripheral Blood	7	91		2
	Peritoneal Smear	5	81		14
	Pericardial Smear	2	95		3
Average	Peripheral Blood	7	90		2.5
	Peritoneal Smear	2	73		25
	Pericardial Smear	2	86		12

*These cells appeared to be ordinary circulating mononuclears rather than the phagocytic cells mentioned in the text.

Experiment 2.—Because of the fact that the peritoneal reaction might be considered possibly to be the result of a species difference, it was decided to repeat the experiment, using autogenous blood from the heart. Because of the small size and delicacy of the mice, it was thought preferable to use rats for this purpose. Accordingly 5 rats were subjected to the following measures: After aseptic preparation a cardiac puncture was carried out and 1 ml. of blood withdrawn. The blood was immediately injected into the peritoneum and the animals were sacrificed at the end of 5 days. The mode of death was by a blow on the head followed by bleeding. Average peripheral blood eosinophilia at this time was 1.16 per cent, of the peritoneal smears 25.2 per cent, of pericardial smears 12.4 per cent (Table II).

Experiment 3.—In order to confirm the results of Experiments 1 and 2, it was decided to repeat these on a slightly different basis, limiting the study to the more critical phase of the reaction, i.e., from 24 to 120 hours following injection. One other difference was introduced. In this experiment the blood

TABLE III. REACTION OF MOUSE TO 1 ML. UNCOAGULATED HUMAN BLOOD INJECTED INTO PERITONEUM
(Average Values for 6 Mice in Each Period)

TIME OF SACRIFICE	PERIPHERAL BLOOD					PERITONEAL SMEARS		
	TOTAL W.B.C.	POLYMORPHONUCLEAR (PER CENT)	LYMPHOCYTE (PER CENT)	MONONUCLEAR (PER CENT)	EOSINOPHIL (PER CENT)	POLYMORPHONUCLEAR* (PER CENT)	LYMPHOCYTE (PER CENT)	EOSINOPHIL (PER CENT)
24 hour	5,225	62.8	32.5	.2	1.5	57.3	38.3	5
48 hour†	2,658	49.7	50.3		0	52.3	38.7	9
72 hour	4,800	36.3	57.4	2	4.3	22	63.2	14.8
120 hour	6,300	32	63.4		4.6	2.3	80.4	17.3

*Human polymorphonuclears excluded from count.
†No visible blood in peritoneal cavity. No human polymorphonuclears seen in peritoneal films, from this time on.

TABLE IV. REACTION OF MOUSE TO 1 ML. HEPARINIZED HUMAN BLOOD INJECTED INTRAPERITONEALLY

TIME OF SACRIFICE	PERIPHERAL BLOOD					PERITONEAL SMEARS*		
	TOTAL W.B.C.	BAND (PER CENT)	SEGMENTED (PER CENT)	LYMPHOCYTE (PER CENT)	EOSINOPHIL (PER CENT)	POLYMORPHONUCLEAR (PER CENT)	LYMPHOCYTE (PER CENT)	EOSINOPHIL (PER CENT)
3 day	6,100	2.5	19.5	70.5	7.5	10	66	24
5 day	7,500	3.5	46.5	40	10	22	66	12
7 day	24,000	1.5	13	78	7	6	90	4
10 day	10,600	4.0	13.5	78	4.5	19	77	4
10 day	17,600	3.5	13	79	3	12	80	8

*No human type polymorphonuclears were observed in any slide.

TABLE V. REACTION OF MOUSE TO 1 ML. CITRATED HUMAN BLOOD INJECTED INTRAPERITONEALLY

TIME OF SACRIFICE	PERIPHERAL BLOOD					PERITONEAL SMEARS*		
	TOTAL W.B.C.	BAND (PER CENT)	SEGMENTED (PER CENT)	LYMPHOCYTE (PER CENT)	EOSINOPHIL (PER CENT)	POLYMORPHONUCLEAR (PER CENT)	LYMPHOCYTE (PER CENT)	EOSINOPHIL (PER CENT)
3 day	6,100	1	41	55	3	34	45	21
5 day	9,425	7	45.5	41	6.5	63	33	4
7 day	15,325	4	22	66	8	9	86	5
10 day	10,850	5	20	70	5	21	68	11
10 day	9,750	5.5	38.5	55	1	11	86	3

*No human type polymorphonuclears were observed in any slide.

from the donor was injected before clot formation had occurred, but no anticoagulant was used. Twenty-four healthy white pedigreed mice were used. Six were sacrificed in each lot, beginning at 24 hours, and thereafter every 24 hours through the 120th hours. The results, as indicated by Table III, bear out the findings previously indicated.

It was thought desirable to check the mice in Experiment 3 for the development of antihuman cell antibodies. Accordingly in each of these animals a preparation of human cells against mouse serum was set up according to the method described by Wright.[30] In the entire group agglutination and hemolysis through 72 hours were the same as for controls, but by the end of 120 hours about half the animals showed varying degrees of either hemolysis or agglutination. There was no evident relationship to the level of eosinophilia.

Experiment 4.—In this experiment the human blood was rendered incoagulable by the addition of heparin. Five mice were injected in the usual manner and were sacrificed at days 3, 5, 7, and 10 (Table IV). Observations were essentially the same as in those treated with coagulated blood.

Experiment 5.—Five mice were treated with human blood to which sterile sodium citrate had been added and were sacrificed and studied as before on days 3, 5, 7, and 10 (Table V). Once more the reaction, both in peripheral blood and peritoneal smears, was of the same character and essentially the same degree. It may be noted that in experiments 4 and 5 the donor cells seem to have persisted in the peritoneum for a longer time than in those experiments in which no anticoagulant was used.

DISCUSSION

Experiments 2 and 3 were carried out to try to determine if the peritoneal eosinophilia was the result of a local or a general hypersensitivity. The results from the injection of autogenous blood into rats would indicate that some other mechanism is at work. Likewise the serological tests on mice which had received human blood seem to support the same inference—that the appearance of local eosinophilia following the injection of human blood into the peritoneum is not closely related to antibody formation. Indeed, the three- to five-day period is usually considered to be short of the time required for the development of hypersensitivity. The point is re-enforced by the discovery of pleural eosinophilia in human beings who have experienced aseptic hemothorax.

Experiments 4 and 5 indicate that neither heparin nor sodium citrate significantly affects the reaction of the peritoneum to blood. They also substantiate the impression that the reaction is independent of the presence of fibrin, though not of fibrinogen. These experiments further indicate that blood, whether clotted or unclotted, whether treated with heparin or with citrate, produces a peritoneal reaction. Indeed the injection of blood into the peritoneum did not fail in a single instance to produce a reaction which was fairly characteristic at any given time.

These observations may explain why patients with effusions of blood into their peritoneum or pleura frequently present a polymorphonuclear leukocytosis, and why frequently one finds in the fluid drawn from these cavities evidence of

mild cellular reaction. Particularly, this study may cast some light on the occasional fluid that presents large numbers of eosinophils. In such cases if microscopy does not reveal red cells, a benzidine test may be of interest. Possibly, but not certainly, serous membrane reaction to blood may explain the fluids one encounters clinically which seem to fall in a vague zone between exudates and transudates and which probably should be distinguished as intermediate zone fluids.

Further studies are in process to determine what portion or portions of the blood produce reactions of the type noted.

SUMMARY

1. The injection of blood into the peritoneum of mice produces a cellular reaction characterized both locally and systemically by early polymorphonuclear leukocytosis, a later lymphocytosis, and still later by a rather persistent eosinophilia. Changes in the peripheral blood seem to precede those in peritoneal smears by about 24 hours. A special characteristic of the peritoneal cellular reaction is the appearance of large mononuclear phagocytes which contain an iron-bearing pigment.

2. Reaction is the same regardless of whether the blood is clotted, unclotted, heparinized or citrated.

3. The reaction appears to be common to all serous membranes, as demonstrated in these experiments in the pericardium and peritoneum, and as suggested by clinical studies of fluids from the pleura.

4. The reaction cannot be one of hypersensitivity, since autogenous blood evokes it, and since the reaction to heterogenous blood is seemingly independent of antibody formation.

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ASSAY OF INTRINSIC FACTOR PREPARATIONS: COMPARISON OF THE HEPATIC UPTAKE OF RADIOACTIVE Co^{60}-B_{12} WITH THE HEMATOPOIETIC RESPONSE IN PERNICIOUS ANEMIA

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AT PRESENT the only method acceptable to the Anti-Anemia Preparations Advisory Board of the U. S. Pharmacopeia for evaluating the potency of intrinsic factor preparations has been the measurement of reticulocyte and red cell responses in patients with pernicious anemia in relapse.[1] It is becoming increasingly difficult to employ this method: It cannot be used on patients in remission, and untreated patients with pernicious anemia are becoming less common in medical centers since diagnosis and treatment are usually carried out by local practitioners. Moreover, the hematological assay may impose hardships upon patients severely ill from pernicious anemia, and completion of the tests may be impossible because of the risks involved in the use of preparations of unknown activity while withholding effective drugs for several weeks.[2, 3] Physicians conducting these prolonged and often nerve-wracking experiments are also subject to great responsibility and strain. The need for an easier and more satisfactory technique is universally recognized.

Three methods based upon the use of radioactive Co^{60}-labeled vitamin B_{12} have recently been proposed for the assay of intrinsic factor activity.

The first, described by Heinle, Welch, and associates,[4] is based upon the determination of the fecal excretion of radioactive vitamin B_{12} in patients with pernicious anemia, following the ingestion of a standard test dose of 0.5 μg Co^{60}-B_{12} of high specific activity, and then determining the decrease in the fecal excretion of Co^{60}-B_{12} upon addition of an effective intrinsic factor preparation.

The second, described by Schilling,[5] measures the increase in the urinary excretion of Co^{60}-B_{12} in patients with pernicious anemia when a potent intrinsic factor concentrate is added to a standard oral dose of 2 μg Co^{60}-B_{12} followed by a "flushing" dose of 1,000 μg of nonradioactive vitamin B_{12} intramuscularly.

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The third, described recently by us,[6-9] is based upon determining the uptake of radioactivity by the liver, when a potent intrinsic factor preparation is given orally to a patient with pernicious anemia together with a standard dose of 0.5 to 2 μg radioactive Co^{60}-B_{12}. The radioactivity is recorded externally over the liver region by means of a scintillation counter. With this test, in normal individuals, the ingestion of radioactive vitamin B_{12} results in the accumulation of the radioactivity in the liver, which rises progressively and usually comes to a plateau between the sixth and tenth day. The uptake can be determined best by measurements of radioactivity made on the sixth and seventh day. In patients with pernicious anemia, in remission or relapse, the absence of intrinsic factor from the stomach results in no or only a negligible passage of vitamin B_{12} through the intestinal membrane and deposition in the liver. Therefore, the hepatic uptake of Co^{60}-B_{12} is nil or negligible in these cases. However, when normal gastric juice or a potent intrinsic factor preparation from the animal stomach is added to the same dose of vitamin B_{12}, the B_{12}-absorption-promoting effect of intrinsic factor results in the absorption of some Co^{60}-B_{12} in the small intestine and its deposition in the liver where it can be detected with the scanning technique just described. By determining in patients with pernicious anemia in remission the increment of radioactivity over the liver following the ingestion of a standard dose of radioactive vitamin B_{12} to which was added the unknown material to be tested for intrinsic factor activity, an expression of the potency of the material may be obtained, based upon the radioactivity counts per minute over the liver.

All three of the isotope techniques can be successfully applied to measure intrinsic factor activity. Each has its virtues and difficulties. What is most important, however, is that all of them can be used on patients with pernicious anemia in remission and apparently also on totally gastrectomized patients.[14-16] This greatly facilitates the assay of intrinsic factor activity and relieves the patient as well as the physician from the hardships of the hematological method.

None of the isotope techniques has been accepted as yet by the Anti-Anemia Preparations Advisory Board of the U. S. Pharmacopoeia as a substitute for the classical method, due to the lack of comparative investigations showing that the isotope techniques are fully equivalent to the classical hematological methods. The purpose of this paper is to present evidence that a good correlation exists between results obtained from the assay of intrinsic factor preparations with the classical method of hematopoietic responses and that using the measurement of the hepatic uptake of radioactive vitamin B_{12}.[8]

MATERIALS AND METHODS

Fourteen different active and inactive intrinsic factor concentrates prepared from animal stomach* were assayed both by hematological assay method and the hepatic uptake

*The intrinsic factor preparations were obtained through the courtesy of Dr. Kenneth W. Thompson, Vice-President, Organon, Inc., Dr. John B. Jewell, Medical Director, Ayerst Laboratories, Drs. Elmer Alpert, Nathaniel Ritter, and Charles Rosenblum of Merck & Company, Inc., Dr. Warren M. Cox, Jr., Director of Nutrition Research, Mead Johnson & Co., Mr. H. J. Anderson of Cudahy Laboratories, and Dr. Ezra Levin, President, Viobin Co.

technique. Hematological studies on nine preparations (64301, 65301, XXI-D-C-a, XVII-D-C-c, I-M-31, I-M-32, G-2, 63301, and 62301) were done by five other investigators.* When submitted for the hepatic uptake measurement, these preparations were identified merely by numbers and we had no prior knowledge of the results of the hematological studies or of the activity of the preparation. The results of both techniques were correlated only after we submitted the results of the isotope assay to the coordinators. This assured an objective evaluation of results. Five other preparations (89697-1, Ay-5310, II-D-2, P-160 SF, and V-IF) were assayed hematologically in our institution.† The hematological assays were performed in each instance by the classical method of reticulocyte and red cell responses in keeping with the requirements of the Anti-Anemia Preparations Advisory Board of the U. S. Pharmacopoeia.[10] The results were compared with standard figures of optimal reticulocyte and red cell responses at various blood levels as published by the Board.[10]

Eight of the preparations used (62301, 63301, G-2, V-IF, XVII-D-C-c, XXI-D-C-a, P-160 SF, and Ay-5310) were active on hematological assay, while five others were inactive, either as a result of technical error in obtaining material from the proper source (preparations 64301 and 65301), or because they represented more or less inactive fractions or extracts processed from the gastric mucosa (preparations 8969-1, I-M-31, and I-M-32). Finally, one preparation (II-D-2) showed only an equivocal activity on hematological assay. Each intrinsic factor preparation studied was used in the dose recommended. We tried to keep doses of intrinsic factor preparations used in the isotope study as near as possible to the daily dose as used in the hematological assay. However, the doses of B_{12} used in both test procedures had to be different (5-15 μg on hematological assay, and 0.5 to 1.0 μg on isotope assay).

The measurements of the hepatic uptake of Co^{60}-B_{12} were done on ten patients with pernicious anemia (seven in remission, two in relapse, and one in both relapse and remission) according to the technique described previously.[8] The general outline of the method used was as follows:

A scintillation counter without collimator and with a 1" × ½" Sodium Iodide Thallium crystal and a Dumont photomultiplier tube and a scaler with precise control of sensitivity and high voltage were used. Counts were taken at a voltage of 1,000 volts, with a background of 60 to 90 counts per minute. Between the sixth and tenth days following the ingestion of a single dose of 1 or 2 μg radioactive vitamin B_{12} of specific activity of 0.212 microcurie per microgram B_{12}, or a single dose of 0.5 μg Co^{60}-B_{12} of specific activity 0.770 microcurie per microgram B_{12},‡ counts were taken over three to four anterior, anterolateral and mid-lateral skin projections of the liver, and four to six areas of the left, mid, and lower abdomen, whereby the counter was held strictly perpendicular to the surface of the organ tested in direct contact with the skin, with the patients in the supine position. Radioactivity was measured on two consecutive days, calculated above background, and corrected for the efficiency of the scaler with the use of a dried Co^{60}-B_{12} standard, and averaged for each day of study. The hepatic and residual abdominal areas were calculated separately. From these multiple averages the mean figure of the hepatic uptake was derived and calculated per 1 microcurie of Co^{60}. This value was checked against the mean figure of the residual abdominal counts, which served as control. If the radioactive material was retained in the bowel due to constipation, a high average abdominal count was obtained which equaled or exceeded that over the liver. In this instance the hepatic count was masked by the scattered radiation from the radioactive material retained within the intestine. A strong purgative (an ounce of castor oil or 15 Gm. of Epsom salt), as well as an enema, were given under these circumstances, and additional counts were taken on two consecutive days two to three days later.

The rate of disappearance of radioactivity from the liver is slow, so that the averaged counts over the liver vary within reasonably narrow limits during the first two to three

*The investigators whose names are listed in Table I generously allowed us to use their data in our paper. Their courtesy is deeply appreciated.

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‡Generously supplied by Dr. Charles Rosenblum of Merck & Co., Inc., Rahway, N. J.

weeks, in patients with pernicious anemia in remission.[8] Moreover, deposition of radioactive vitamin B_{12} in the liver shows an additive character, so that similar increments in radioactivity over the liver area are obtained in the same individual following repeated ingestion of identical doses of radioactive material.[8] This permits the use of the averaged hepatic and abdominal counts at the end of the first counting period as a base line for the next experimental period; from this, further increments in radioactivity occurring after ingestion of a subsequent dose of radioactive material are calculated. Accordingly, eight to ten days after the administration of the initial dose of Co^{60}-B_{12} alone, the same dose of radioactive material was given again orally to the same experimental subject, but this time with an intrinsic factor preparation to be tested. The counts were taken again in the same way and hepatic uptake calculated as previously. Since the dose of radioactive vitamin B_{12} ingested is far below the maximum permissible dose of 3 μc of Co^{60} which may be safely contained within the human,[11] no hazard to individuals tested occurs, even if the tests are repeated several times on the same person.

TABLE I. HEMATOLOGICAL RESPONSES TO INTRINSIC FACTOR PREPARATIONS IN PATIENTS WITH PERNICIOUS ANEMIA IN RELAPSE

I. F. PREPARATION	INVESTIGATOR*	CASE (INITIALS OR NUMBER)	DAILY ORAL DOSE		RED BLOOD CELLS 10^6 IN CU. MM.				RETICULOCYTES PEAK IN %	EVALUATION OF RESPONSE†
			I. F. IN MG.	VIT. B_{12} IN μG	INITIAL COUNT	COUNT AFTER 2 WEEKS	COUNT AFTER 3 WEEKS	INCREMENT AFTER 3 WEEKS		
62301	T. D. S.	B. B.	40.0	15.0	2.42	3.14	3.75	1.33	19.1	Optimal
	T. D. S.	G. W.	40.0	15.0	2.49	3.19	3.75	1.26	17.4	Optimal
	J. R. P. H.	J. F.	40.0	15.0	1.12	2.43	2.85	1.73	17.1	Optimal
63301	T. D. S.	C. T.	40.0	15.0	2.48	3.27	3.78	1.30	20.3	Optimal
(4635)	T. D. S.	G. D.	40.0	15.0	2.18	2.91	3.41	1.23	19.7	Optimal
	T. D. S.	B. W.	40.0	15.0	2.33	3.19	3.61	1.28	18.8	Optimal
	D. L.	63940	40.0	15.0	1.78	3.58	3.19	1.41	2.9	Suboptimal
G-2	T. D. S.	A. C.	40.0	15.0	2.38	3.67	4.09	1.71	18.4	Optimal
	T. D. S.	J. J.	40.0	15.0	2.58	3.68	4.31	1.73	20.3	Optimal
V-IF	Our group	J. M.	200.0	15.0	2.05	3.15	3.60	1.55	13.0	Near optimal
XVII-D-C-c	F. H. B.	J. S.	10.0	5.0	1.60	2.80		1.20‡	25.0	Optimal
XXI-D-C-a	F. H. B.	A. C.	10.0	5.0	1.80	2.20		0.40‡	15.1	Positive
I-M-32	W. B. C.	N. N.	5.0	5.0	1.62	1.97§			4.0	Negative
I-M-31	W. B. C.	K. M.	1.0	5.0	1.61	1.48‖			5.8	? Weakly positive
	T. D. S.	1 case	1.0	5.0						Negative
64301	T. D. S.	6 cases	16.0–40.0	5.0–15.0						Negative
65301	T. D. S.	6 cases	40.0	15.0						Negative

*The results of these hematological assays were generously supplied by:
Dr. Tom D. Spies — T. D. S. Dr. Frank H. Bethell — F. H. B.
Dr. Jack Rheingold and Dr. Pearl Holly — J. R. P. H.
Dr. Daniel Laszlo — D. L. Dr. William B. Castle — W. B. C.

†According to the standards of the United States Pharmacopoeia Anti-Anemia Preparations Advisory Board.

‡Increment after two weeks.

§After 12 days.

‖After 13 days.

The results of the hepatic uptake measurements were evaluated with reference to data obtained by the use of an intrinsic factor preparation of known activity. This preparation representing the intrinsic factor concentrate portion of 1 U. S. P. oral unit of "Vitamin B_{12} with intrinsic factor concentrate,"* when given to a patient with pernicious anemia in remission together with 1 μg Co^{60}-B_{12} of 0.21 μc radioactivity, resulted in hepatic uptake of 346 to 649 averaged counts above background per minute and per 1 microcurie Co^{60} (Table I, preparation No. 4635, at a dose of 60 mg.).

*Kindly supplied by Dr. Kenneth W. Thompson, Organon, Inc.

All of the intrinsic factor preparations supplied for the isotope tests (with the only exception of the preparation XVII-D-C-c) did not contain any crystalline B_{12} added, and according to manufacturers' statements were devoid of any detectable B_{12} activity on microbiological assay.

TABLE II. MEASUREMENTS OF HEPATIC UPTAKE OF RADIOACTIVITY IN 7 PATIENTS WITH PERNICIOUS ANEMIA IN REMISSION FOLLOWING ORAL ADMINISTRATION OF INTRINSIC FACTOR PREPARATION TOGETHER WITH RADIOACTIVE Co^{60}-B_{12}

		HEPATIC UPTAKE MEASUREMENT						
					AVERAGE HEPATIC UPTAKE ABOVE BACKGROUND			
I. F. PREPARATION	CASE	DOSE OF B_{12} IN μG	DOSE OF Co^{60} IN μC	DOSE OF I. F. IN MG.	C.P.M.	C.P.M. PER 1 μC Co^{60}	EVALUATION OF ACTIVITY	HEMATOLOGICAL RESPONSE
62301	P. M.	1.0	0.21	40.0	65	305	Active	Optimal*
63301	P. M.	1.0	0.21	40.0	46	216	Active	Optimal or
(4635)	W. B.	1.0	0.21	40.0	41	193	Active	suboptimal*
	W. B.	1.0	0.21	60.0	78	346	Active	
	P. M.	1.0	0.21	60.0	131	616	Highly active	
	A. S.	1.0	0.21	60.0	138	649	Highly active	
G-2	G. S.	2.0	0.42	40.0	164	436	Highly active	Optimal
V-IF	G. S.	0.5	0.385	66.6	119	309	Active	Optimal† or
	G. S.	0.5	0.385	132.0	67	174	Active	near optimal*
P-160 SF	P. M.	0.5	0.385	26.0	106	276	Active	Near optimal‡
	G. S.	0.5	0.385	40.0	75	195	Active	
	P. M.	0.5	0.385	50.0	92	239	Active	
	B. M.	0.5	0.385	50.0	25	65	Slightly active	
	M. V.	0.5	0.385	50.0	82	213	Active	
	M. McD.	0.5	0.385	100.0	108	281	Active	
XVII-D-C-c	A. S.	5.4	0.63	10.0	12	19	Trace of activity	Optimal*
XXI-D-C-a	P. M.	0.5	0.385	10.0	74	192	Active	Positive*
I-M-32	W. B.	1.0	0.21	14.0	15	71	Slightly active	Negative*
	A. S.	1.0	0.21	15.0	21	99	Slightly active	
	P. M.	1.0	0.21	5.0	33	155	Slightly active	
I-M-31	P. M.	1.0	0.21	1.0	0	0	Not active	Negative*
	P. M.	1.0	0.21	8.0	0	0	Not active	
64301	A. S.	1.0	0.21	40.0	0	0	Not active	Negative*
65301	P. M.	1.0	0.21	40.0	0	0	Not active	Negative*
89697-1	A. S.	0.5	0.385	100.0	0	0	Not active	Negative§

*See Table I.
†See Table III.
‡See Fig. 1 and Table III.
§See Fig. 2 and Table III.

RESULTS

Nine intrinsic factor preparations (listed in Table I) were assayed hematologically by other investigators on their own patients with pernicious anemia in relapse. The isotope assays of these nine preparations were done in our institution separately on other patients with pernicious anemia in remission (Table II). Five other intrinsic factor preparations were tested in our institution by both methods on the same patients with pernicious anemia in relapse (Table III).

TABLE III. EVALUATION OF THE ACTIVITY OF 5 DIFFERENT INTRINSIC FACTOR PREPARATIONS BY THE MEASUREMENT OF THE HEMATOLOGICAL RESPONSE AND HEPATIC UPTAKE OF RADIOACTIVE Co^{60}-B_{12} IN 3 PATIENTS WITH PERNICIOUS ANEMIA IN RELAPSE

PATIENTS' INITIALS, SEX AND AGE	INTRINSIC FACTOR PREPARATION	DAILY ORAL DOSE		HEMATOLOGICAL ASSAY							ISOTOPE ASSAY					
				RED BLOOD CELLS × 10^6 IN C.MM.												
				COUNT			INCREMENT			RETICULOCYTES PEAK %	EVALUATION OF RESPONSE	SINGLE ORAL DOSE			AVERAGE HEPATIC UPTAKE ABOVE BACKGROUND		EVALUATION OF ACTIVITY
		I. F. IN MG.	B_{12} IN μG	INITIAL	AFTER 2 WEEKS	AFTER 3 WEEKS	AFTER 2 WEEKS	AFTER 3 WEEKS				I. F. IN MG.	B_{12} IN μG	Co^{60} IN μC	C.P.M.	C.P.M. PER 1 μC Co^{60}	
M. H. f. 64	89697-I	100.0	15.0	2.50	2.32		0.18			2.8	Negative	100.0	0.5	0.385	0	0	Not active
	Ay-5310	150.0	15.0	2.40	3.49		1.09			11.2	Optimal	150.0	0.5	0.385	234	608	Highly active
A. L. f. 70	V-IF	200.0	15.0	1.52	2.75	3.35	1.23	1.83		32.7	Optimal	200.0	0.5	0.385	56	146	Slightly active
F. N. f. 64	II-D-2	40.0	15.0	1.85	2.22		0.37			6.4	Slight	40.0	0.5	0.385	58	151	Slightly active
	P-160 SF	100.0	15.0	2.25	3.35	3.55	1.00	1.30		10.9	Near optimal	100.0	0.5	0.385	111	289	Active

The Assay of Intrinsic Factor Preparations by the Hematological Method on Patients With Pernicious Anemia in Relapse and the Isotope Assay of These Preparations on Patients With Pernicious Anemia in Remission.—

These studies were done on twelve preparations listed in Table II, whereby all the isotope assays were performed in our Laboratory and the hematological studies were done on nine preparations elsewhere (marked by one asterisk), and on three patients in our institution. As shown in Table II, seven of these preparations (62301, 63301, G-2, V-IF, P-160 SF, XVII-D-C-c, and XXI-D-C-a) were active on hematological assay, whereas five others (I-M-32, I-M-31, 64301, 65301, and 89697-I) were hematologically inactive. The detailed data of their hematological activity are listed in Tables I and III.

Some of the intrinsic factor preparations were tested on several patients by the hematological and isotope methods. Thus, preparation 63301 (4635) was tested hematologically by two different investigators on four patients, and by the isotope method in our institution in five tests on four other patients. Preparation I-M-31 was assayed by both methods on two patients. Preparation 62301 was tested by the hematological method on three patients by two different investigators, preparations 64301 and 65301 on six patients each, and preparation G-2 on two patients. Preparation P-160 SF was assayed on six different patients and I-M-32 on three by the isotope method. The hematological and isotope studies were done frequently using various doses of the same intrinsic factor preparation on various patients. The results obtained were generally concordant, with the allowance made for individual responsiveness.

Of seven hematologically active preparations (Table II), six (with the exception of preparation XVII-D-C-c) were found active when tested with the isotope method of hepatic uptake measurements. On the other hand, of five hematologically inactive preparations (Table II), four were not active also on isotope assay. Therefore, of twelve intrinsic factor preparations tested with hematological and isotope methods, identical results were obtained with both techniques in ten. Some discrepancy was noted in regard to two out of twelve preparations tested. The electrophoretically prepared fraction XVII-D-C-c,* although active hematologically, caused only a very slight hepatic uptake of Co^{60}-B_{12}; and the electrophoretically processed fraction I-M-32, which showed only slight reticulocyte response in a patient with pernicious anemia in relapse when tested by the hematological method, has shown slight activity in three different patients when tested by the isotope method of hepatic uptake measurements. The possible reasons for these discrepancies will be discussed.

Comment.—Very satisfactory correlation was obtained with twelve intrinsic factor preparations listed in Table II between hepatic uptake measurement of Co^{60}-B_{12} on pernicious anemia patients in remission, and that obtained with the classical method of reticulocyte and red cell response on patients with pernicious anemia in relapse. Whenever material tested showed a definitely positive hepatic uptake of Co^{60}-B_{12} (preparations 62301, 63301 (4635), G-2, V-IF, P-160 SF, and XXI-D-C-a) the preparation was also active hematopoietically. On the other

*Kindly supplied by Dr. Kurt G. Stern of Brooklyn Polytechnic Institute and Dr. Kenneth W. Thompson, Organon, Inc.

hand, whenever the hepatic uptake of Co^{60}-B_{12} with a preparation tested was zero (preparations I-M-31, 64301, 65301, and 89697-I), the preparation was also absolutely inactive on patients with pernicious anemia in relapse when tested hematologically or showed a questionable activity (preparation I-M-31). The significance of this positive correlation is enhanced by the objectivity of this study in which we had no prior knowledge of the results obtained by other investigators with the hematological method (Table I), before we submitted our results to the coordinator.

In only two cases in which the hepatic uptake measurements showed a slight and equivocal activity, the correlation with the results of the hematological assay was not complete. Preparation I-M-32 was slightly active by three separate isotope tests on three patients with pernicious anemia in remission, but gave a negative result on hematological assay in a patient with pernicious anemia in relapse. Another preparation, XVII-D-C-c, showed definite activity on hematological assay, but only a trace of activity when tested by the hepatic uptake measurement. In this latter case, however, the preparation used contained a surplus of nonradioactive B_{12} which raised the total dose of B_{12} to 5.4 μg, instead of the usual dose of 0.5 to 1.0 μg used in our method. Since at this dose the efficiency of intestinal absorption of vitamin B_{12} falls to about 20 per cent of the ingested dose,[12] a decrease in the absorption of Co^{60}-B_{12} as well would result. This could be the reason for the slight activity of this preparation in the isotope test, as compared to its potency in the hematological assay.

From data listed in Table II, it appears that whenever the intrinsic factor preparation gives a clear-cut positive or negative response with the isotope test on a patient with pernicious anemia in remission, it would also be active or inactive, respectively, in the hematological assay on the patient with pernicious anemia in relapse.

Assay of Intrinsic Factor Preparations by Both Hematologic and Isotope Methods on the Same Patients With Pernicious Anemia in Relapse.—

Five intrinsic factor preparations were tested by both isotope and hematological methods on three patients with pernicious anemia in relapse (Table III).

The preparations 89697-1 and Ay-5310 were assayed on Patient M. H. (Fig. 1), the preparations II-D-2 and P-160 SF on Patient F. N. (Fig. 2), and the preparation V-IF on Patient A. L. (Table III). Since these data allow better comparison of both methods, they will be reported more in detail.

CASE 1.—M. H., a 64-year-old woman (No. 6205, Flower and Fifth Avenue Hospitals), had typical pernicious anemia in the second relapse with classical history of progressive weakness, loss of appetite, and anemia. Physical examination disclosed light lemon yellow tint of skin, smooth and somewhat atrophic tongue, loss of vibratory sense from the toes to the ankles, positive Romberg, no free hydrochloric acid in the gastric juice after subcutaneous histamine injection, and a megaloblastic bone marrow. The thyroid was slightly enlarged, and the basal metabolic rate was +21 per cent. Serum bilirubin was 1.27 mg. per 100 ml. There was no occult blood in the stools on several examinations. X-ray series of the gastrointestinal tract and barium enema were negative. Blood counts on admission were as follows: erythro-

cytes, 1.26 million per cubic millimeter; hemoglobin, 5.5 Gm. per cent; hematocrit, 17.0 per cent; MCV, 135.0 cu. μ; MCH, 43.7 $\mu\mu$; MCHC, 32.3 per cent; leukocytes, 3,800 per cubic millimeter; differential, 48 per cent lymphocytes; reticulocytes, 2.1 per cent.

After the patient received a blood transfusion of 500 ml., which increased her red blood cell count to 2.30 million and her hemoglobin to over 8 Gm. per cent, hepatic uptake measurements were started. First, a tracer dose of 1 μg Co^{60}-B_{12} containing 0.21 μc Co^{60} was given. Eight days later a similar dose of Co^{60}-B_{12} together with 100 mg. of intrinsic factor concentrate 89697-1 was administered. Twelve days later another tracer dose of 1 μg Co^{60}-B_{12} was given, this time with 116 mg. of intrinsic factor concentrate 4635.

As shown in Fig. 1, the administration of radioactive B_{12} alone, as well as with 100 mg. of the intrinsic factor preparation 89697-1, did not result in any hepatic uptake of radioactivity or in any reticulocyte response, indicative of no demonstrable intestinal

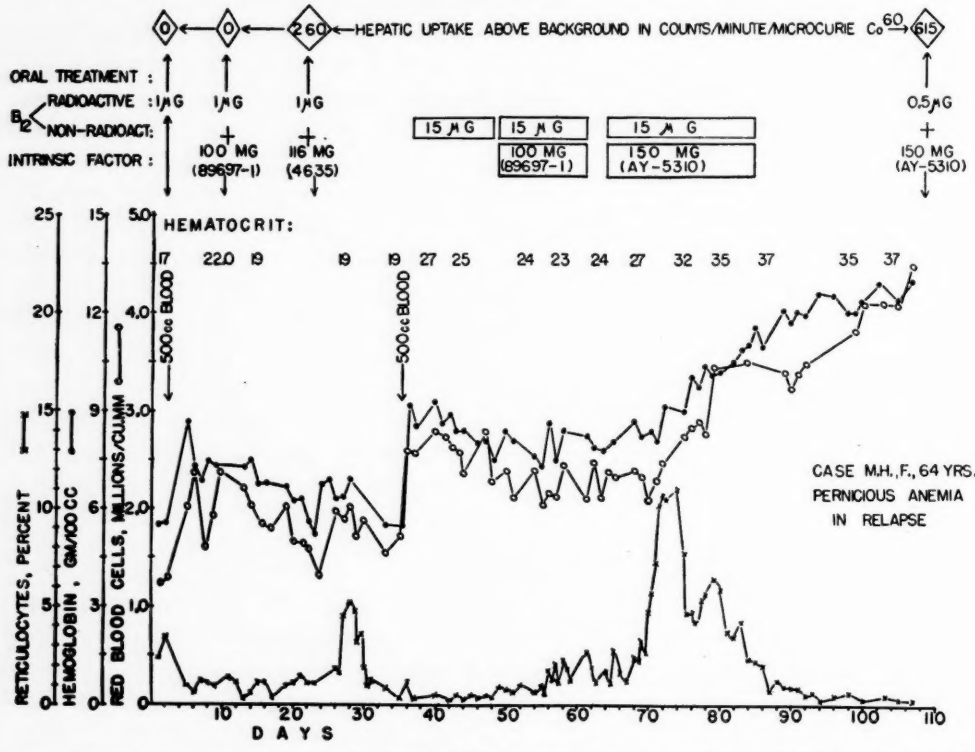

Fig. 1.—Case 1. Assay of three intrinsic factor preparations (89697-1, 4635, and AY-5310) by both hematologic and isotope methods in a patient with pernicious anemia in relapse.

absorption of vitamin B_{12} under these circumstances. However, when the same tracer dose of 1 μg Co^{60}-B_{12} was given together with 116 mg. of the intrinsic factor concentrate (of a known potency, equivalent to that of intrinsic factor material contained in 2 U.S.P. units of "Intrinsic factor concentrate with vitamin B_{12}"), a definite hepatic uptake was observed which amounted to an average of 260 counts per minute per 1 μc Co^{60}. The absorption of at least a part of the ingested 1 μg of vitamin B_{12} under these circumstances was also proved hematologically by a slight reticulocyte response of 6 per cent which occurred on the sixth day and was associated with a slight, though very brief rise in red cells, hematocrit, and a sense of well-being in the patient.

After the second blood transfusion of 500 ml., vitamin B_{12} alone was given daily by mouth at a dose of 15 μg for 11 days. This had no effect on the blood status or clinical symptoms. Thereafter, a similar dose of 15 μg of vitamin B_{12} daily was given by mouth for twelve days, together with 100 mg. of intrinsic factor concentrate 89697-1. Paralleling the negative results which were obtained previously in the measurement of the hepatic uptake, no significant hematopoietic response was observed. Although there was a reticulocytosis of 2 to 3 per cent, the red cells and hematocrit slowly decreased.

Then a similar oral daily dose of 15 μg vitamin B_{12} was given together with 150 mg. of a third intrinsic factor concentrate (Ay-5310). A sharp classic reticulocyte rise occurred, with a peak of 11.6 per cent on the ninth day. This may be considered practically optimal since the expected peak of reticulocytes is about 13 per cent when starting with a level of 2.40 million red blood cells. An almost maximal rise in red cells, hemoglobin and hematocrit values was also obtained during three weeks of continued treatment. The red blood cells increased from 2.40 million to 3.49 million, the hemoglobin from 8.1 to 11.0 Gm. and the hematocrit rose from 24.5 to 37.5 per cent (Fig. 1). A month later the red blood cells increased to 4.48 million and the hemoglobin to 13.0 Gm. per cent without any further treatment. Then the patient received the same tracer dose of 1 μg Co^{60}-B_{12} plus 150 mg. of the hematopoietically active intrinsic factor concentrate (Ay-5310). The hepatic uptake was positive (615 counts per minute per 1 μc Co^{60}) which was in accord with the hematopoietic activity of the preparation demonstrated previously in this patient.

Case 2.—F. N., a 64-year-old woman (No. 6462, Flower and Fifth Avenue Hospitals), had typical pernicious anemia in first relapse with a history of dizziness, loss of appetite, and excessive weakness for one year. The physical examination disclosed pallor of the skin, general disorientation and prostration, absence of knee and ankle jerks, and no pathologic findings in other organs. Blood count on admission was as follows: 1.89×10^6 erythrocytes; 7.2 Gm. per cent hemoglobin; 24 per cent hematocrit; 3,500 leukocytes, with predominance of lymphocytes, and 0.7 per cent reticulocytes. There was no free hydrochloric acid on gastric analysis after histamine. Bone marrow was megaloblastic. Bilirubin was 1.4 mg. per cent. Blood chemistry was normal.

The hepatic uptake measurement following oral administration of 0.5 μg Co^{60}-B_{12} containing 0.385 microcurie Co^{60} revealed no uptake of radioactivity by the liver indicative of defective intestinal absorption of vitamin B_{12} typical for pernicious anemia (Fig. 2). A few days later the same test was repeated after addition of 40 mg. intrinsic factor preparation II-D-2 to the same dose of 0.5 μg Co^{60}-B_{12}. Two days later oral treatment was started in the form of 15 μg B_{12} orally plus 40 mg. II-D-2 intrinsic factor preparation in daily single doses for fourteen days.

The measurement of the hepatic uptake of radioactivity performed on the sixth and seventh day after administration of 0.5 μg Co^{60}-B_{12} plus 40 mg. II-D-2, revealed slight activity of this preparation, as shown by an uptake of 151 c.p.m. per 1 μc Co^{60}. In line with this result, the hematological response obtained was also moderate and certainly suboptimal. It consisted in a slight clinical improvement, a reticulocyte peak of 6.4 per cent, rise in erythrocytes from 1.85 to 2.22×10^6 and that of hemoglobin from 7.0 to 9.0 Gm. per cent after two weeks of the treatment.

Then the oral treatment with 15 μg B_{12} and 40 mg. of II-D-2 intrinsic factor preparation was stopped, and six days later a single dose of 0.5 μg Co^{60}-B_{12} was given orally again, this time with 100 mg. P-160 SF intrinsic factor preparation. Two days later the daily oral treatment with 15 μg crystalline B_{12} and 100 mg. of the same P-160 SF intrinsic factor preparation was started in two divided doses and was continued daily for the duration of the next three weeks.

The measurement of the hepatic uptake six and seven days after administration of Co^{60}-B_{12} with 100 mg. P-160 SF has shown a positive uptake of 289 c.p.m. per 1 μc Co^{60}, indicative of definite activity of this preparation. This was confirmed by the near optimal

hematopoietic response following oral treatment with this combination, and was characterized by a reticulocyte peak of 10.9 per cent on the eleventh day, rise in erythrocytes from 2.25 to 3.55 × 10^6, hemoglobin from 7.8 to 11.7, and hematocrit from 26 to 40 per cent after three weeks of this treatment (Fig. 2). This was followed by a complete clinical remission.

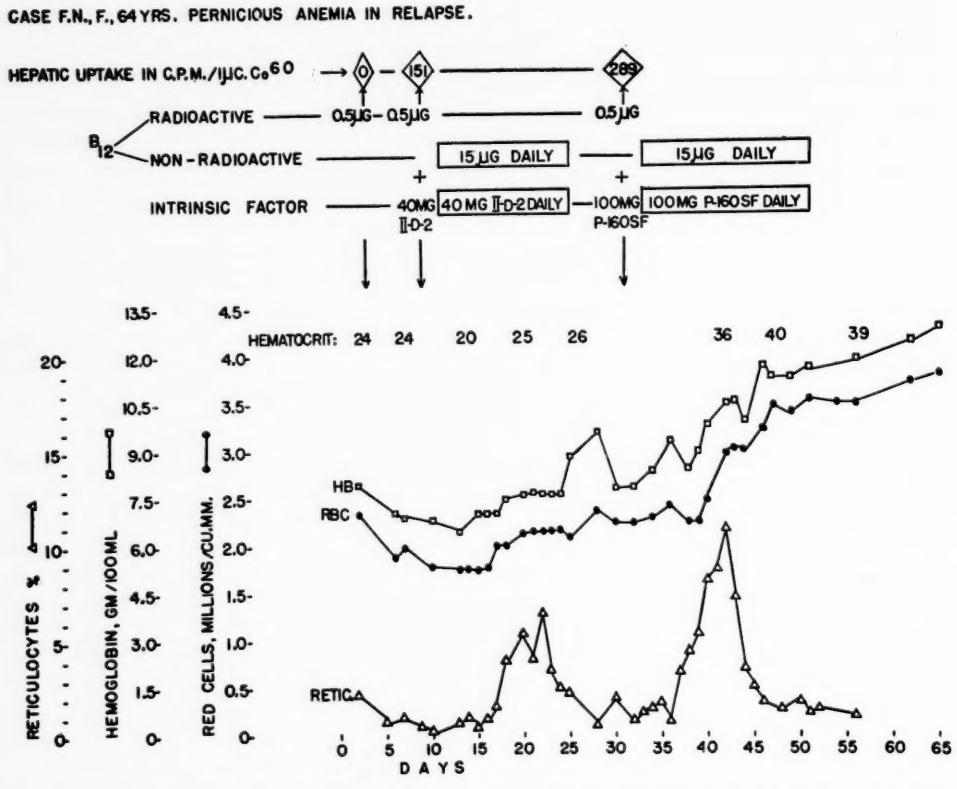

Fig. 2.—Case 2. Assay of two intrinsic factor preparations (II-D-2 and P-160 SF) by both hematologic and isotope methods in a patient with pernicious anemia in relapse.

Case 3.—A. L., a 70-year-old woman (No. 11471, Flower and Fifth Avenue Hospitals), had typical pernicious anemia with a history of progressive weakness, loss of appetite, heart palpitation, and pallor of six weeks' duration. On physical examination the following were noted: pallor with yellowish tinge, atrophic tongue, systolic murmur over the entire precordium, loss of position sense in lower extremities, and no other detectable abnormalities in other organs. Blood count was as follows: 1.84 × 10^6 erythrocytes; 5 Gm. per cent hemoglobin; 4,200 leukocytes with 44 per cent lymphocytes. There was no free hydrochloric acid in gastric juice after histamine injection. Bone marrow was highly megaloblastic. Icteric index was 30; Van den Bergh, direct negative; fragility of red cells: initial hemolysis—0.42 per cent sodium chloride, complete hemolysis—0.34 per cent sodium chloride.

The hepatic uptake measurement following oral administration of 0.5 μg Co^{60}-B_{12} containing 0.385 microcuries Co^{60} revealed no uptake of radioactivity by the liver indicative of defective intestinal absorption of vitamin B_{12} and typical for pernicious anemia. A few days later the same test was repeated after addition of a single dose of 200 mg. V-IF intrinsic factor preparation. The measurement of the hepatic uptake of radioactivity performed six and seven days later revealed an uptake of 146 c.p.m. per 1 μc Co^{60}, indicative of activity of this preparation.

Thereupon, three weeks' oral therapy was started in the form of 15.0 μg crystalline B_{12} and 200 mg. V-IF intrinsic factor preparations daily (in two divided doses). An optimal hematopoietic response was obtained, characterized by a reticulocyte peak of 32.7 per cent on the eighth day, rise in erythrocytes from 1.52 to 3.35 × 10^6, hemoglobin from 5.4 to 9.5 Gm. per cent, and hematocrit from 13 to 32 per cent at the end of three weeks. The hematopoietic response was associated with and followed by a complete clinical remission.

Comment.—In the assay of intrinsic factor preparations a good correlation was obtained on patients with pernicious anemia in relapse between the clear-cut positive or negative results of the measurement of the hepatic uptake of Co^{60}-B_{12} and the hematopoietic response to the same preparation in the same individual (Table III). Preparations Ay-5310 and P-160 SF were definitely active on isotope assay and also gave optimal or nearly optimal hematopoietic responses in the same patients (Figs. 1 and 2). Moreover, preparation P-160 SF was more active on the isotope assay on the same patient than preparation II-D-2, which showed only slight hepatic uptake, and so was it also on the hematopoietic assay on the same individual (Fig. 2). Preparation 89697-1 was definitely negative on the isotope assay, and also showed no hematopoietic activity on the same patient (Fig. 1).

When the measurement of the hepatic uptake of Co^{60}-B_{12} showed a slight activity of the preparation, the correlation between both tests did not appear to be as complete. In two different cases and at different doses two intrinsic factor preparations (V-IF and II-D-2) gave similar moderate values of hepatic uptake. However, when tested hematologically on the same two patients, one (II-D-2) gave a moderate, but the other (V-IF) an optimal, hematopoietic response.

DISCUSSION

The data listed in this paper establish a good correlation between the results obtained in the assay of commercial intrinsic factor preparations by the hepatic uptake measurement of Co^{60}-B_{12} and the classical technique of hematologic responses. It was obtained irrespective of whether the assays were performed on the same or on different individuals, provided the results of the isotope study were clear-cut, i.e., clearly positive or clearly negative.

Whenever the results of the isotope test were equivocal (slightly positive) the correlation was not reliable and the results of the hematological assay could be negative (preparation I-M-32, Table II), moderately positive (preparation II-D-2, Table III), or optimally positive (preparation V-IF, Table III).

The explanation of this discordance probably lies in different doses of vitamin B_{12} used with the same dose of intrinsic factor preparations when tested by the isotope and hematological methods. It appears from our previous work[13] that the results of the isotope assays depend very much on the correct ratio between the dose of B_{12} and that of intrinsic factor used. Too much or too little of intrinsic factor preparation may vitiate the intestinal absorption of the minute dose of radioactive B_{12} used in these assays, and change entirely the result of the test. Also, the content of large amounts of B_{12}-binding, but

hematologically inert, substances in crude intrinsic factor preparations may decrease the absorption of Co^{60}-B_{12} and detract from the sensitivity of the isotope test.[13]

It is quite possible, therefore, that the same intrinsic factor preparation which shows only slight activity in one isotope test would prove much more active if tested by the same method under more optimal conditions. This factor apparently is much less important in the hematological assay. The dose of B_{12} used in hematological assay is many times larger than that in the isotope test, so that there is always some surplus of B_{12} which is not absorbed.[12] This feature of the isotope techniques, which operate with minute oral doses of B_{12}, is responsible, we think, for some of the equivocal results obtained. However, when the isotope test gives a clearly positive or negative result, then the positive correlation with the result of the hematological assay is regularly observed.

Thus, the usefulness of the measurement of the hepatic uptake of radioactive B_{12} in the assay of intrinsic factor preparations was clearly demonstrated by complete agreement obtained between the results of this method and the hematopoietic responses to eleven different intrinsic factor preparations from commercial sources. The obvious advantages of our isotope method are: (1) its applicability to patients with pernicious anemia in remission, thus circumventing most of the difficulties encountered in obtaining and handling untreated cases of pernicious anemia or those in relapse[2, 3]; (2) independence of the procedure upon the cooperation of the patient, which is needed in other (excretory) isotope methods[4, 5]; (3) ease and simplicity of the scanning procedure; (4) directness of the evidence obtained.

SUMMARY AND CONCLUSIONS

The potency of fourteen different active and nonactive intrinsic factor preparations from hog stomach was tested independently by the technique of the measurement of the hepatic uptake of Co^{60}-B_{12} in eight patients with pernicious anemia in remission and by the classic hematologic method of reticulocyte and red cell response in twenty patients with pernicious anemia in relapse, with the help of other investigators. In three patients with pernicious anemia in relapse the assay of five intrinsic factor preparations was performed by both the measurement of the hepatic uptake of radioactive Co^{60}-B_{12} and the classical method of hematopoietic responses.

In three instances, in which the isotope assay gave equivocal results and showed only a slight activity of the preparation tested, the correlation with the hematopoietic responses was not satisfactory. This was apparently due either to the not optimal ratio between Co^{60}-B_{12} and intrinsic factor dose in the isotope assay, presence of nonradioactive B_{12} in the preparation, which decreased the absorption of radioactive B_{12}, or, finally, to the excessive binding of B_{12} by inert materials present in some of the crude intrinsic factor preparations.

In all other instances in which the hepatic uptake measurements showed clear-cut positive or negative results, the agreement between the results of the

isotope assay and the hematological classical method was complete. This absolute concordance was obtained under rigid experimental conditions in testing eleven different commercial intrinsic factor preparations from hog stomach.

In the screening of the intrinsic factor preparations the measurement of the hepatic uptake of radioactive Co^{60}-B_{12} in patients with pernicious anemia in remission compares favorably with the method of measuring the reticulocyte and red cells responses on patients with pernicious anemia in relapse.

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THERMAL RANGE OF ACTIVITY OF ANTIBODIES ELUTED FROM THE RED CELLS IN AUTOIMMUNE HEMOLYTIC ANEMIA

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IT IS well known that antibodies against red blood cells react best with their respective antigens at certain temperature ranges. Some reactions are best demonstrated at 37° C., whereas, in others, lower temperatures are required. In general, "naturally occurring" red cell antibodies show greater "affinity" for the antigen at low temperatures. On the other hand, the antibodies that are produced after active immunization react equally well or even better at body temperature.[1, 2, 3, 4, 5, 7, 8, 9, 10, 16] Exceptions to this general rule, however, have been reported.[6, 11, 12, 13] Whether there is any fundamental difference between the so-called naturally occurring hemagglutinins and hemolysins and the immune ones is not certain. The former may very well be the result of inapparent "heterogenetic" immunization.[14]

In autoimmune hemolytic anemia, antibodies can almost always be found attached to the red cell surface and quite often free in the serum as well. In most cases the serum of the patient reacts best with normal erythrocytes in the cold.[15, 19, 21, 22] Nevertheless, the thermal amplitude of the fixed antibodies does not seem to have been investigated so far. Only Kidd[17] states that these antibodies after having been eluted off the red cell surface are able to sensitize normal erythrocytes equally well at all temperatures between 4° and 37° C. He does not report, however, whether the eluates were titrated at different temperatures.

In the present work, an attempt was made to determine the optimal thermal range of activity of the antibodies eluted from the red cells of a number of patients with acquired autoimmune hemolytic anemia.

MATERIALS AND METHODS

Twenty-nine eluates derived from the red cells of fifteen patients with autoimmune hemolytic anemia were used for the present study. In 11 of these patients the disease was "idiopathic" and, in 4, secondary to chronic lymphocytic leukemia. All patients had a strongly positive Coombs' test. The dissociation of the antibody from the red cells was achieved by exposure to low pH, high salt concentration, or to high temperature as described in a previous communication.[18] Especial effort was made to avoid contamination of the

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eluate with serum proteins. Blood was drawn into warm glass containers and placed immediately in a water bath at 37° C. Acid-citrate-dextrose solution was used as anticoagulant. The red cells were separated from the plasma by centrifugation and washed 5 to 10 times with large volumes of normal saline warmed to 37° C. Between each successive washing, the red cell-saline mixture was incubated at 37° for at least 10 minutes. After the last washing the packed cells were kept in the refrigerator for several hours and then examined by naked eye for the presence of autoagglutination. If there was no visible clumping, elution was carried out. Otherwise the cells were subjected to further washing.

Two separate twofold serial dilutions of each eluate were prepared. One was placed in a water bath at 37 ± 2° C., and the other in an ice bath at 3 ± 1° C. A 2 per cent suspension of the test cells was prepared and brought to the corresponding temperature before being added to the dilutions of the eluate. Washed red cells from normal individuals of group O Rh negative (cde/cde), either untreated or trypsinized, were used as test cells. If the cells were used untreated, they were incubated for two hours with the eluate dilution, washed with saline at the appropriate temperature, and then reacted with Coombs' antiglobulin serum. The Coombs' tests were performed at 37° C. If trypsinized red cells were used, the test tubes were examined directly for agglutination after a two-hour incubation period. The degree of agglutination was graded from + to ++++.

Absorption experiments were performed in the following way: Equal portions of each eluate were mixed with arbitrary but constant amounts of normal washed packed red cells. After incubation for a given period of time at 37 ± 2° C., 22 ± 3° C., and 3 ± 1° C., the cells were removed by centrifugation at the corresponding temperature and the titers of the supernatants determined against normal trypsinized cells at 37° C. Trypsinization of the red cells was performed by the method described previously.[18]

RESULTS

Table I shows a typical example of two different eluates that were tested directly against trypsinized normal red cells at 37° and at 3° C. In Fig. 1 the titers of the eluates were plotted against the temperature and it is readily apparent that the titers of all were lower at 3° than at 37° by from 1 to 7 tubes.

TABLE I. TITRATION OF TWO ELUATES AGAINST TRYPSINIZED NORMAL ERYTHROCYTES AT 37° AND AT 3° C. (INCUBATION PERIOD 3 HOURS)

	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	SALINE CONTROL
Eluate I at 37°	+++	+++	+++	++	++	+	–	–	–
Eluate I at 3°	++	+	+	–	–	–	–	–	–
Eluate II at 37°	++++	++++	+++	+++	++	+	+	–	–
Eluate II at 3°	++	±	–	–	–	–	–	–	–

The agglutination was also weaker in the cold and took longer to develop. There was no apparent difference in that respect between the antibodies derived from the "idiopathic" and those from the secondary form of the disease. Results were the same whether the titers were determined against trypsinized cells or by the indirect Coombs' technique. The method used for elution had no influence on the optimal thermal range of activity of the obtained eluate. It was also noted (Fig. 1) that the sera of most of these patients contained cold agglutinins at the time when the blood was drawn for elution.

Typical absorption experiments are shown in Tables II and III. For the absorption tests either a relatively large number of red cells were incubated with the eluate for a short period of time (Table II) or a small number of red cells

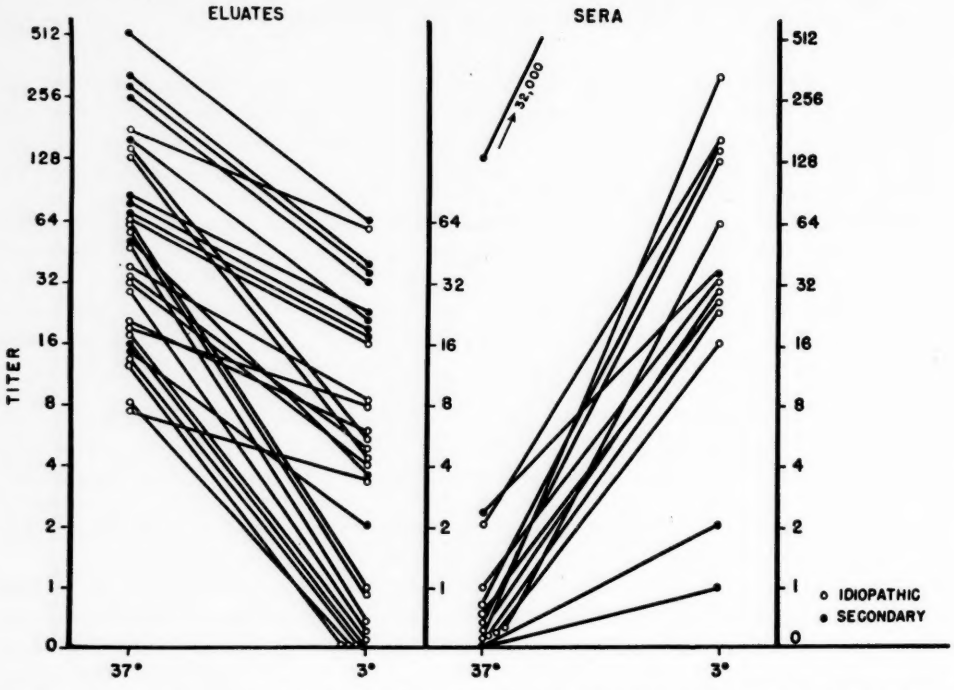

Fig. 1.—Effect of temperature on the titer of antibodies eluted from the red cells and found free in the sera of patients with autoimmune hemolytic anemia.

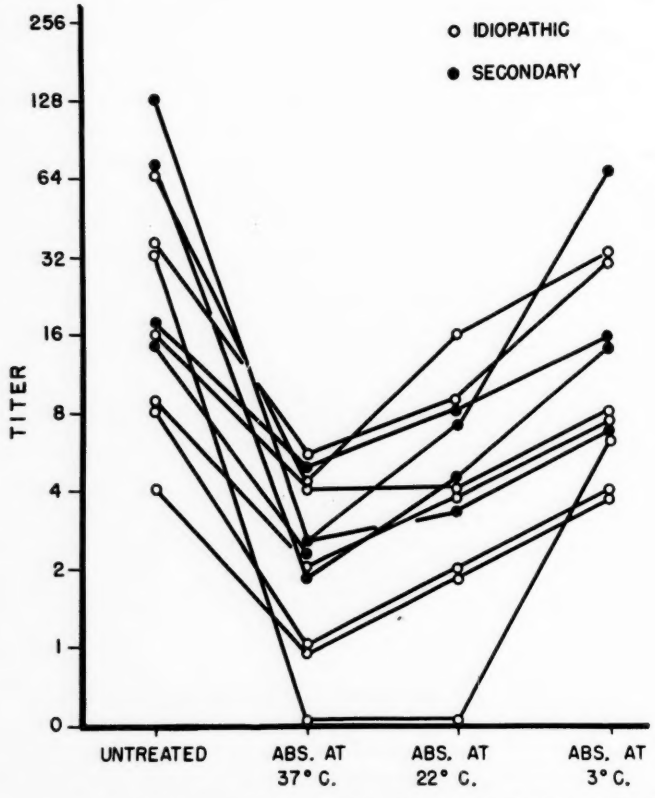

Fig. 2.—Absorption of eluates at 37° C., 22° C., and 3° C.

for a long period of time (Table III). Results were about the same, and indicated that the reaction was not only more rapid at 37° but that each red cell could actually bind more antibody at body temperature than in the cold. Fig. 2 shows the results of a number of absorption experiments performed by both methods. Coombs' tests were performed on the cells used for the absorptions. The reaction was clearly stronger on the cells that were incubated with the eluates at 37° C.

TABLE II. ABSORPTION EXPERIMENT
(Equal amounts of the eluate were absorbed with constant amounts of red cells over a short period of time)

	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	SALINE CONTROL
Before absorption	++++	++++	++++	+++	+++	+	+	–	–
After abs. at 37°	++	+	–	–	–	–	–	–	–
After abs. at 22°	+++	++	+	–	–	–	–	–	–
After abs. at 3°	++++	++++	+++	++	+	–	–	–	–

TABLE III. ABSORPTION EXPERIMENT
(Equal portions of the eluate were absorbed with a small amount of red cells over a period of 4 hours)

	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	SALINE CONTROL
Before absorption	+++	+++	+++	+++	+	±	–	–	–
After abs. at 37°	+++	++	+	–	–	–	–	–	–
After abs. at 22°	+++	++	+	–	–	–	–	–	–
After abs. at 3°	++++	++++	++++	+++	+	–	–	–	–

At 22° C. the behavior of the antibodies varied (Tables II and III and Fig. 2). Some of them appeared to react as strongly at that temperature as at 37°. Others seemed to become attached to the red cells somewhat better at 22° than at 3° but not as efficiently as at body temperature.

DISCUSSION

In this study, it was clearly shown that the antibodies which were eluted from the red cells of a number of patients with autoimmune hemolytic anemia reacted best with the antigen at 37° C.

The first question that arises is whether these results represented the true behavior of the original antibody or whether the elution procedure altered the antibody in some way. This question cannot be answered with certainty. We believe, however, that the antibody could not have been altered very drastically since it retained its ability to react with red cells. This, along with the fact that the thermal behavior was the same regardless of the method of elution used, strongly indicates that the above results represented the optimal thermal range of activity of the original material.

In most cases of autoimmune hemolytic anemia with a positive direct Coombs' test, indicating antibody adherent to the red cells, free antibodies are also detectable in the serum of the patient as well. Usually, the sera of these patients react best with normal red cells in the cold, even though some reaction takes place at 37° C. in many cases. The question then arises whether the free

"cold" antibody and the antibody that is attached to the red cells represent one and the same material with a wide thermal range of activity or whether they are two different antibodies. If the positive Coombs' test in our cases were due to a cold antibody with a wide thermal range of activity, then that antibody should continue to react best in the cold after its elution from the red cell surface. This did not prove to be the case in the studies reported here. In all the cases that were tested, a warm variety of antibody was eluted from the red cells, thus indicating that the antibody had become attached to the red cells of the patient in the warm environment of the body. On the other hand, the cold antibodies remained free in the serum (Fig. 1).

The mechanism of production of the various antibodies that are encountered in autoimmune hemolytic anemia is obscure. It has been suggested that they are produced in response to a viral antigen or to a modification of the antigenic structure of the red cell by a virus or some other agent.[19, 20] This would imply that they were actually the product of active immunization. They have, as generally agreed, one thing in common with the immune iso- and hetero- red cell antibodies, namely, that they are incomplete in nature. The results of the present work indicate that at least in the cases tested an antibody was present that had a second property in common with the immune antibodies, namely, the ability to react best with red cells at physiologic temperatures, i.e., at 37° C.

SUMMARY

Antibodies eluted from the red cells of 15 cases of autoimmune hemolytic anemia were found to react with normal erythrocytes better at 37° C. than in the cold. The possible significance of this finding is discussed.

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VARIABLE MANIFESTATIONS OF PLASMA THROMBOPLASTIN COMPONENT DEFICIENCY

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IT IS now well established that plasma thromboplastin component (PTC),[1] also known in the British literature as Christmas factor,[2] is essential for the formation of blood thromboplastin.[3, 4] Absence or marked diminution of PTC causes a severe syndrome clinically indistinguishable from classical hemophilia.† The latter disorder is still widely attributed to the lack of antihemophilic factor (AHF), equally necessary for the production of thromboplastin.

If the syndromes are fully developed, differentiation between hemophilia and PTC deficiency is feasible by relatively simple laboratory procedures. It has been shown that $BaSO_4$ adsorbs PTC but not AHF,[5] and that serum contains only PTC.[5] Addition of these two reagents (absorbed normal plasma containing AHF and normal serum containing PTC) to the patient's blood will selectively correct the abnormal clotting and serum prothrombin times. In some instances, however, the clotting anomalies and clinical manifestations may be of a much milder nature. The thromboplastin generation test,[3] which is technically more complicated, may then be of aid in establishing the correct diagnosis, even if the clotting and serum prothrombin times are within normal limits.

Hemorrhagic manifestations in both conditions, hemophilia and PTC deficiency, are usually encountered only in males who are considered to possess one pathologic gene responsible for the particular disease. The heterozygous female (conductor) is asymptomatic. Attempts to demonstrate some regular expression of the pathologic genes in the conductor by determination of the clotting time and the prothrombin consumption test have not yielded clear-cut results.[7-14]

This paper reports three families with PTC deficiency. In the first, the asymptomatic mother of the severely ill propositus showed a definitely decreased thromboplastin generation, although her serum prothrombin time was normal. Laboratory findings, identical with those obtained in this conductor, were observed in three male members of the second family who exhibited only

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†The controversy whether hemophilia is caused by a real deficiency of AHF or a functional deficiency due to the presence of an inhibitor[6] falls outside the scope of this paper. Our considerations are essentially applicable to either one of these interpretations.

mild hemorrhagic manifestations. In the third family, the conductor showed a normal thromboplastin generation, although her offspring was afflicted with a severe type of the disease.

These data reveal the value of the thromboplastin generation test for the demonstration of an otherwise nonidentifiable expressivity of the pathologic gene in mild types of the syndrome and irregularly in the conductors.

CASE REPORTS

FAMILY 1.—The propositus, S. L., a 26-year-old white man, had suffered from repeated attacks of severe bleeding into his muscles and various joints as well as from hematuria since his first year of life. Marked deformities of his knee joints were immediately noticeable on physical examination. He was diagnosed as a typical case of hemophilia but, in 1954, was sent to our laboratory for the differential diagnosis between classic hemophilia and "Christmas disease." As can be seen from Table I, the patient exhibited the characteristic laboratory features of severe PTC deficiency. His abnormal clotting and serum prothrombin times were normalized by addition of normal serum and by hemophilic plasma, but not by the supernatant of normal plasma absorbed with $BaSO_4$. The thromboplastin generation test (Fig. 1) demonstrated the complete absence of any thromboplastic activity. When normal serum was substituted for the patient's serum, a normal generation resulted.

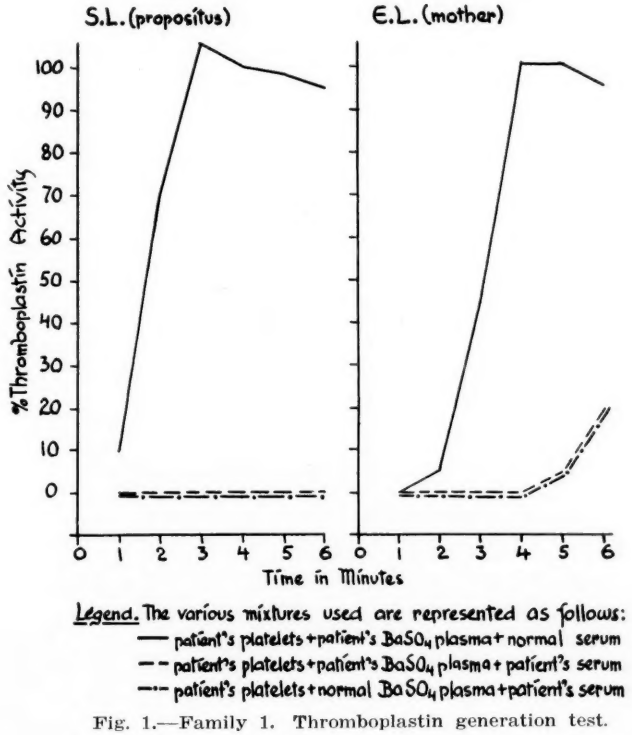

Fig. 1.—Family 1. Thromboplastin generation test.

The propositus' mother, Mrs. E. L., aged 52 years, had given birth to four sons. Two had died from severe bleeding in infancy, and one was clinically and hematologically entirely normal. This latter finding established the heterozygosity of Mrs. L. for the abnormal gene. Two of her brothers had also died from "hemophilia." Her husband had never had any disorder suggestive of a hemorrhagic diathesis, and no clotting abnormality

could be detected in our studies. Mrs. L. showed no objective evidence of abnormal bleeding. Her four deliveries were not associated with excessive hemorrhages. However, she recalled having bled profusely after hysterectomy and also after a tooth extraction. The results of the clotting tests in this conductor of the PTC syndrome are compiled in Table I. The clotting time was slightly prolonged, but the serum prothrombin time was definitely within normal limits. The thromboplastin generation test revealed a marked abnormality; approximately only 20 per cent of the value considered to be still normal are found. This disturbance of the thromboplastin generation could be corrected, selectively and completely, by substitution of her serum by normal serum. Thus, in this asymptomatic carrier, the thromboplastin generation test revealed a definite PTC deficiency.

TABLE I. RESULTS OF CLOTTING STUDIES IN FAMILY 1

TYPE OF TEST	PROPOSITUS, S. L.	CONDUCTOR, MRS. E. L.
Clotting time (Lee-White) normal: up to 20 minutes	Over 240	23
Prothrombin time, one-stage,[27] in seconds	11.5	11
Two-stage prothrombin concentration[28]	Not performed	Normal (260 Iowa units)
Fibrinogen[29]	Not performed	320 mg. %
Clotting time in minutes after addition* of:		
normal $BaSO_4$ plasma	Over 240	20
normal serum, aged 48 hr.	10	11.5
hemophilic plasma	12	Not performed
Serum prothrombin time (60 minutes after clot formation), one-stage procedure, in seconds (normal over 20 sec.)	13.4	35
Serum prothrombin time in seconds after addition* of:		
normal $BaSO_4$ plasma	13.4	37
normal serum, aged 48 hr.	39	60
hemophilic plasma	37	Not performed
Test for circulating anticoagulant[30]	Negative	
Thromboplastin generation test[3]	Abnormal (see Fig. 1)	

*Reagent 0.1 ml. to tested blood 0.9 ml.

TABLE II. RESULTS OF CLOTTING STUDIES IN FAMILY 2

TYPE OF TEST	R. S.	D. S.	M. S.
Clotting time in minutes	20	17	18
Clotting time in minutes after addition of:			
normal $BaSO_4$ plasma	18	16	16
normal serum	9	10	6
Plasma prothrombin time in seconds	11	11.5	11
Serum prothrombin time (60 minutes after clot formation) in seconds	31	33	36
Test for circulating anticoagulant	Negative		
Thromboplastin generation test	Abnormal (see Fig. 2)		

TABLE III. RESULTS OF CLOTTING STUDIES IN FAMILY 3

TYPE OF TEST	PROPOSITUS, M. Q.	CONDUCTOR, MRS. E. Q.
Clotting time in minutes	45	8
Clotting time in minutes after addition of:		
normal $BaSO_4$ plasma	35	Not performed
normal serum	8	Not performed
Plasma prothrombin time in seconds	12.8	12.2
Serum prothrombin time (60 minutes after clot formation) in seconds	14.0	32.4
Serum prothrombin time in seconds after addition of:		
normal $BaSO_4$ plasma	14.0	Not performed
normal aged serum	50.0	Not performed
Test for circulating anticoagulant	Negative	
Thromboplastin generation test	Abnormal (see Fig. 3)	Normal (see Fig. 3)

FAMILY 2.—In 1954, three brothers—R. S., aged 27, D. S., aged 16, and M. S., aged 15—were sent to our laboratory for re-evaluation of their "hemophilic" syndrome. In all three individuals the clinical manifestations were relatively mild, consisting of hemorrhages into the skin and muscles following slight trauma or dental and surgical procedures. Spontaneous bleeding was never observed in two, and only the oldest brother may once have had mild bleeding into the knee joint. However, no residual deformities were noticeable. The results of the pertinent laboratory tests are compiled in Table II and Fig. 2.

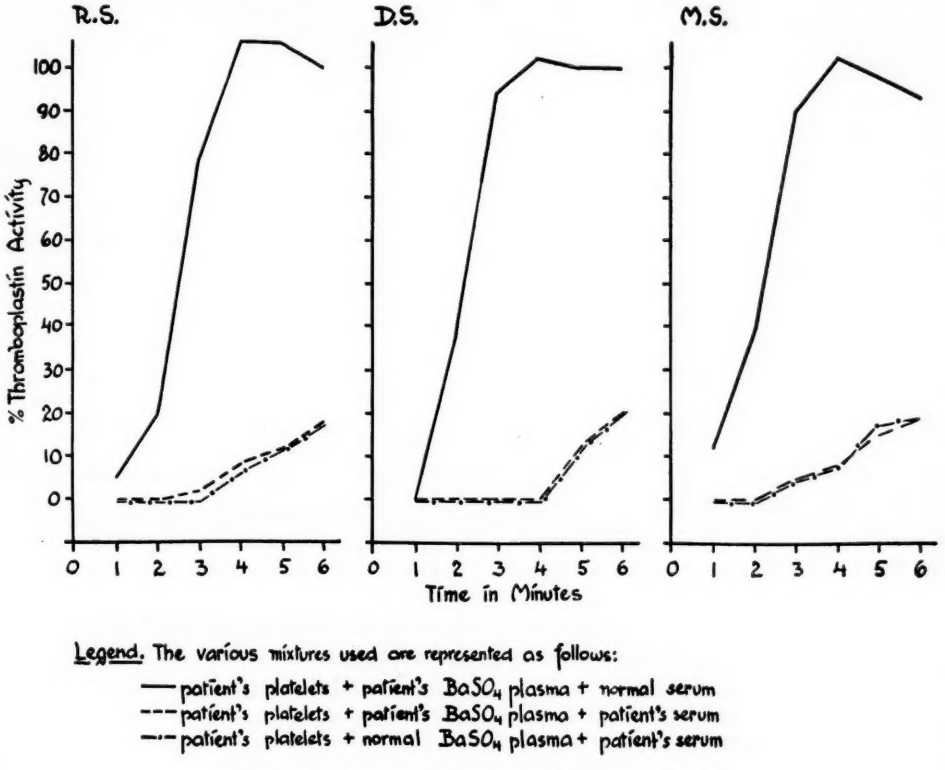

Fig. 2.—Family 2. Thromboplastin generation test.

As can be seen, the clotting and the serum prothrombin times were within the normal ranges. The marked shortening of the clotting times by serum, but not by $BaSO_4$ plasma, is noteworthy, but, in our experience, addition of aged serum has a distinct, although slight, shortening effect on the clotting times of normal as well as of hemophilic individuals.

The thromboplastin generation test was distinctly abnormal in all three siblings. Substitution of the patients' sera by either normal serum or a PTC preparation, obtained from serum by the method of White and associate,[5] rectified the thromboplastin generation. Using normal serum instead of the patients' $BaSO_4$ plasma did not influence the results. Thus, the definite diagnosis of PTC deficiency in these three individuals could be established only by means of the thromboplastin generation test.

The mother, Mrs. C. S., representing the conductor state, showed no clinical or hematological abnormalities. Her thromboplastin generation was entirely normal. One of her three brothers was reported to have a hemorrhagic diathesis similar to that of her sons.

FAMILY 3.—The propositus, M. Q., aged 5½ years, had exhibited hemorrhagic manifestations since his birth. He bled profusely from the umbilicus and showed repeated attacks of bleeding into his muscles and joints. The knee joints were markedly deformed. He received numerous blood transfusions for his ailment. Family history showed the propositus' younger brother had died from "hemophilia," and two of his maternal uncles were known to be severe bleeders but were not available for examination. Mrs. E. Q., the propositus' mother, had never experienced any abnormal hemorrhages. The pertinent laboratory studies are compiled in Table III and Fig. 3. The diagnosis of PTC deficiency was established without difficulty. In contrast to the conductor of Family 1, the conductor in this family revealed no abnormality in her thromboplastin generation, although her offspring suffered from the severe form of the disease.

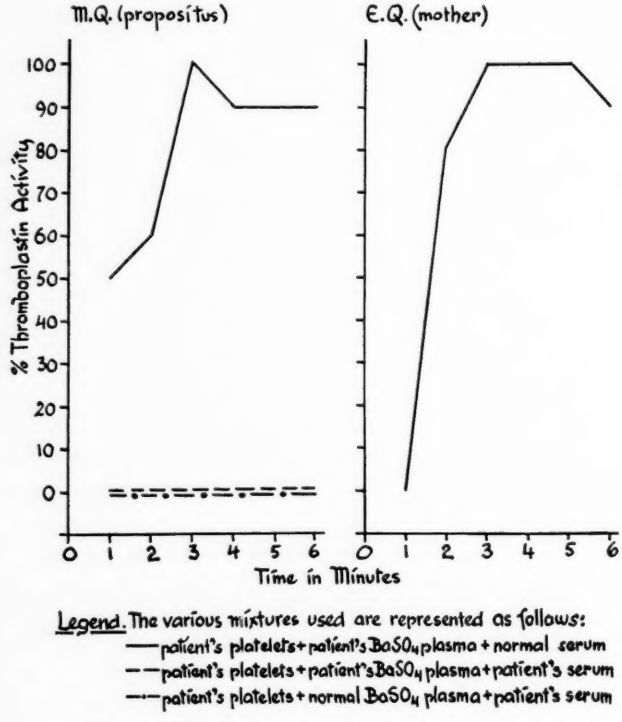

Fig. 3.—Family 3. Thromboplastin generation test.

DISCUSSION

Our studies indicate that in clinically mild PTC deficiency the thromboplastin generation test may be a very sensitive laboratory procedure, leading to the correct diagnosis. This observation confirms the findings of Bergsagel and co-workers,[15] who also reported two individuals with such a syndrome in whom only the thromboplastin generation was abnormal, whereas all the other clotting tests were noncontributory. Similarly, the thromboplastin generation test revealed a clotting defect in the mother of our first patient with severe PTC deficiency. Since only substitution of the conductor's serum by normal serum rectified the result of the test, it must be concluded that this

conductor had a clinically latent, but nevertheless definite, PTC deficiency. However, the thromboplastin generation of the two other examined conductors was normal.

These particular data raise two significant questions: (1) Why is the PTC deficiency syndrome mild in some families and severe in others? (2) Why is the heterozygous female clinically asymptomatic, although a PTC deficiency may be demonstrable, whereas the "hemizygous"* male with similar laboratory findings exhibits hemorrhagic manifestations?

Both classic hemophilia and the PTC syndrome are commonly ascribed to the presence of a specific abnormal sex-linked characteristic. Recently it has been emphasized that hemophilia does not always appear as a severe disease but may be present in milder or even subclinical forms.[6, 17, 18] However, family studies demonstrate that the type of hemophilia does not vary within the same kindred over many generations.[18, 19] Brinkhous, Graham, and McLendon[16-18] postulated a gene H which regulates the normal production of antihemophilic factor. In severe hemophilia, this gene is replaced by the abnormal allele h, and in the mild type by another allele designated as h^m. Measurements of plasma AHF activity in such families provide the basis of this multiple allele hypothesis. In classic hemophilia, no AHF is found, but in the milder type a subnormal AHF level is detectable.[16-18]

One is tempted to apply a similar explanation for the variable degree of PTC deficiency in different families, thus postulating various abnormal alleles for the gene responsible for the production of PTC. The constancy of the syndrome within the same sibship is quite conspicuous in Family 2, as may be seen from the almost identical curves depicting the results of the thromboplastin generation test (Fig. 2).

The multiple allele hypothesis fails, however, to explain why the heterozygous conductors in families with severe hemophilia show no decrease in their AHF activity,[16-18] although some but not all of the conductors with mild hemophilia exhibit some definite diminution of this factor.[16] Furthermore, in contrast to these studies, our conductor with an abnormal thromboplastin generation test transmitted a severe type of PTC deficiency to her offspring. However, in the two other families with either severe or mild PTC deficiency the conductor state could not be identified. Bergsagel and associates had similar results.[15]

The fact that the heterozygous female conductor is clinically asymptomatic, in contradistinction to the hemizygous male, has stimulated many investigations. Preceding the delineation of the PTC deficiency syndrome, Merskey and Macfarlane[7] in 1951 determined the clotting and serum prothrombin times in more than 20 known carriers. Although occasionally the prothrombin consumption was slightly abnormal, the conclusion was reached

*In genetic terminology, the term "heterozygous" is used when there is a difference between the members of a pair of genes. Since in the case of sex-linked traits the male has but one representative of a particular locus (on the X chromosome) instead of the usual two, the term "heterozygous" is not applicable. In such instances, the term "hemizygous" has been employed for males.[31] Females, however, may be either "heterozygous" or "homozygous" for this particular gene.

that the results of these tests are too indefinite and inconsistent to be of diagnostic value. Identical findings were obtained by others.[8-14, 20, 21] Owren[13] reported that activation of proconvertin to convertin, which requires thromboplastin, is slower than normal in hemophilia conductors. Stefanini[14] has mentioned that while silicone clotting times and prothrombin consumption tests are normal in female carriers of the hemophilia gene, their plasma fails to correct completely the coagulation defect of the plasma of known hemophiliacs. On the other hand, Brinkhous and co-workers,[17, 18] as previously mentioned, were unable to detect any diminution of AHF activity in the conductors of severe canine and human hemophilia, and Biggs and Douglas[3] observed a normal thromboplastin generation in 7 such individuals. We can confirm the finding of a normal thromboplastin generation in 8 conductors of classic hemophilia.[22] Thus, it appears that the conductor state in true hemophilia cannot be readily identified.

Since the discovery of the PTC factor in 1952 and the recognition that PTC deficiency may be clinically and genetically indistinguishable from AHF deficiency, relatively few studies are yet available of the conductors of this disorder. Without differentiating between severe and mild types in the male offspring, the conductors showed inconstantly an abnormal serum prothrombin time.[2, 20] Essentially, with the use of the prothrombin consumption test, the findings in the conductors of families with the PTC syndrome are the same as those stated for classic hemophilia.

All these data indicate that the abnormal genes by themselves apparently do not produce any alterations in the "heterozygous" state. They require "activation" by some other factor or factors, also probably of genetic nature. The postulated genetic interaction of the abnormal genes and the modifiers may then result in a variable expressivity of the former. The pathologic genes (for AHF and PTC) appear to be located on the X chromosome and the modifier(s) on the Y chromosome. However, since the conductors may irregularly show some expressivity of the pathologic genes, a modifying factor not located on the Y chromosome may also at times be present. It may be argued that if the clinical manifestations depend on the interaction of the abnormal gene and the modifier(s), the observed constancy of the picture in the same family is not adequately explained, since one must expect that these two genetic factors segregate independently. No studies of apparently unaffected brothers of hemophiliacs by means of the thromboplastin generation test are yet available which may establish whether "male conductors" of the hemophilic gene exist.

Such a genetic situation is by no means unique in hematological diseases. In the hereditary syndromes associated with abnormal hemoglobins a variable expressivity of the abnormal genes is frequently observed.[23] For example, it seems established that the sickle-cell trait carrier is heterozygous for the gene for S hemoglobin. However, quantitative electrophoretic measurements of this abnormal hemoglobin show variations from 20 to 50 per cent of the total pigment in such individuals.[24, 25] The percentage of S hemoglobin may,

however, be relatively constant in members of the same family.[26] To explain these individual differences, a genetic modifier of the expressivity of the pathologic gene for S hemoglobin has been postulated.[23, 26]

The significance of modifying factors regulating the expressivity of pathologic genes usually becomes quite apparent when methods are developed which permit quantitative estimation of the basic pathologic alterations. It is for this reason that the very sensitive thromboplastin generation test should perhaps be applied more frequently in future studies of conductors in the various types of the PTC deficiency syndrome.

SUMMARY

Three families exhibiting PTC deficiency syndromes are reported. In one, the asymptomatic mother of the propositus, with clinically severe manifestations, shows a definitely decreased thromboplastin generation, although her serum prothrombin time is normal. Results of clotting studies identical with those obtained in this conductor were observed in three male members of another family who suffer from a mild hemorrhagic diathesis. These data reveal the diagnostic value of the thromboplastin generation test for the demonstration of an otherwise nondetectable expressivity of the pathologic gene responsible for the PTC deficiency syndrome. The genetic situation in the various types of PTC deficiency is analyzed.

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A SIMPLE, SPECIFIC ONE-STAGE PROTHROMBIN ASSAY USING RUSSELL'S VIPER VENOM IN CEPHALIN SUSPENSION

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INTRODUCTION

A ONE-STAGE prothrombin assay is based upon timing the interval between recalcification of a test system and clotting. This time interval depends upon the initial rate of thrombin accumulation in the clotting mixture. This rate measures the prothrombin content of the test plasma only when the rest of the clotting mixture provides a constant, optimal concentration of all other clotting factors, or their equivalent. Nonspecific factors that influence the interval between recalcification and clotting, such as ionic strength, pH, and temperature, must not vary.

Quick's construction of the first one-stage "prothrombin" test[1] was a big step forward in coagulation methodology. This simple test remains a valuable clinical and research tool. However, its value lies partly in that it is not a specific prothrombin test but is sensitive to changes in the proaccelerin (labile factor, plasma Ac globulin) and proconvertin (stable factor, Factor VII, SPCA) content of the test plasma. Furthermore, it is insensitive to changes in prothrombin concentration in the 50 to 100 per cent of normal range.

These difficulties can be surmounted by diluting the test plasma, which increases the sensitivity of the system, and by introducing reagents that contain a high and constant concentration of the nonprothrombin factors. Adsorbed ox plasma provides a high and constant concentration of proaccelerin and fibrinogen. It has been used for years in this laboratory in a one-stage method, the P. and P. method,[2, 3] for the quantitative determination of the combined effect of prothrombin and proconvertin in patients on Dicumarol therapy. The P. and P. method can be converted into a specific prothrombin assay by the addition of a constant concentration of proconvertin in the form of specially prepared prothrombin "free" stored serum.[3]

Fullerton[4] first substituted Russell's viper venom for brain thromboplastin in the Quick test. However, the clotting time using venom was found to vary with the lipoid and/or platelet content of the plasma. Furthermore, it failed to measure the full effect of Dicumarol therapy. This is because the clotting action of viper venom in the presence of lipoid is equivalent to convertin (the combination of thromboplastin and proconvertin) and therefore independent of the proconvertin content of the test plasma.[5, 6]

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Preliminary experiments revealed that the use of Russell's viper venom dissolved in crude "cephalin" suspension in place of brain thromboplastin changed the P. and P. test into a specific one-stage assay of prothrombin alone.[7] Further experience has perfected this technique of prothrombin assay into a method which is superior to the older test with stored serum. The present paper describes the reagents, technique, and standardization of the specific one-stage venom-cephalin assay for prothrombin as it is now used in this laboratory.

MATERIALS AND METHODS

Test Plasma.—Four and five-tenths milliliters of blood is drawn into 0.5 ml. of 0.1 M (3.13 Gm. per cent) sodium citrate dihydrate and centrifuged at 2,500 r.p.m. for 15 minutes. *Standard plasma* for preparing the correlation graph is made by drawing 180 ml. of normal blood into 20 ml. of the same anticoagulant. The plasma, obtained by centrifugation at 30 minutes at 2,500 r.p.m., is stored in small quantities in the liquid frozen state at -20° C.

Dilution Fluids.—The usual dilution of the test plasma is 1:50. The standard plasma must be diluted from 1:10 to 1:500 to prepare the correlation graph. The diluting technique must keep the citrate concentration and ionic strength constant for any dilution within this range. This is done by making two dilutions. The plasma is diluted first to 10 times the final concentration desired (1:5 for the test plasma and up to 1:50 for the standard plasma) in Diluting Fluid I. This consists of 100 ml. of 0.1 M sodium citrate added to 600 ml. of 0.9 Gm. per cent sodium chloride solution and has the same citrate concentration and ionic strength as citrated plasma from blood with a normal hematocrit. A second 1:10 dilution of the various primary dilutions is then made in Diluting Fluid II which consists of 200 ml. of Veronal buffer, 200 ml. of 25.66 mM sodium citrate and 600 ml. of 0.9 Gm. per cent sodium chloride solution.

Crude "Cephalin" Suspension.—This is the acetone-insoluble, ether-soluble fraction of human brain prepared according to Milstone's technique.[8] Four hundred grams of human brain are washed free of blood and membranes and ground with 300 ml. of acetone.* After centrifugation, the acetone is discarded and the residue re-extracted with the same volume of acetone a total of six times. The residue is then extracted with 1,800 ml. of ether overnight at room temperature. The ether is siphoned off and evaporated to dryness by suction at 35° C. The resulting residue is washed twice for 15 minutes with 900 ml. of acetone and then redissolved in 200 ml. of ether. The ether is again evaporated to dryness and the suction continued for 75 minutes. About 9.3 Gm. of a waxy cream-colored to brown residue are suspended in 200 ml. of Veronal buffer. This is centrifuged for 20 minutes at 1,700 r.p.m. and again for 10 minutes at 2,500 r.p.m. The sediment is discarded. The supernatant, which contains 2.8 Gm. per cent of crude cephalin in a milky white suspension, is frozen in small quantities at -20° C. Before use, the melted suspension is diluted 1:30 in veronal buffer to a final concentration of 0.09 Gm. per cent.

Russell's Viper Venom-Cephalin Reagent.—The preparation Stypven† is used. The dried venom is dissolved in the 0.09 Gm. per cent cephalin suspension to a concentration of 1:40,000.

Small portions of this reagent were stored at 37° C., 20° C., 4° C., and -20° C. It proved remarkably stable even at 37° C. There was no deterioration after standing for one day at 20° C., or for several days at 4° C. The reagent could be frozen and thawed repeatedly without loss of activity. It proved easiest to store it at -20° C. in aliquots suitable for one day's use. It can probably stay frozen indefinitely without loss of activity.

Prothrombin (and Proconvertin) Free Bovine Plasma.—The bovine plasma should contain no prothrombin, a high and stable concentration of proaccelerin, and a constant and

*Merck & Company, Inc.
†Burroughs Wellcome & Company, Inc.

stable fibrinogen. The technique for preparing the corresponding reagent for the P. and P. method (filtration of oxalated ox plasma once through 20 per cent and once through 50 per cent asbestos filter pads) leaves traces of prothrombin in the adsorbed plasma. This is unimportant in the P. and P. test but reduces greatly the sensitivity of the venom-cephalin test.

A suitable reagent is obtained as follows: Nine hundred milliliters of ox blood is collected at the slaughter house in 1,000 ml. ice-cold bottles packed in ice and containing 100 ml. of 2.5 per cent potassium oxalate monohydrate. The blood is centrifuged as soon as possible for 30 minutes at 2,500 r.p.m. in a refrigerated centrifuge. The plasma is passed through a 20 per cent asbestos filter pad (filter diameter 15 cm., oxygen pressure about 1 kg. per square centimeter, filter changed for every 500 ml.). The filtered plasma is then adsorbed for 5 minutes at 20° C. with barium sulfate (Baker), 75 mg. per milliliter of plasma, and the barium sulfate removed by centrifugation. The adsorbed plasma is stored in small aliquots liquid frozen at -20° C., in which state it remains stable for long periods.

Veronal Buffer.—This buffer of pH 7.35 and ionic strength 0.154 is made by mixing sodium diethyl barbiturate 11.75 Gm., sodium chloride 14.67 Gm., 0.1 N HCl 400 ml., and distilled water to 2,000 ml.

Calcium Chloride.—A 35 mM solution is made by dilution of a 1 M aqueous stock solution with distilled water.

The test is done by mixing 0.2 ml. of adsorbed bovine plasma, 0.2 ml. of the Russell's viper venom-cephalin reagent, and 0.2 ml. of a dilution of the test plasma in a serologic test tube which is then incubated for 5 minutes in a water bath at 37° C. Then 0.2 ml. of calcium chloride is blown into the tube and the clotting time measured. All tests are done in duplicate.

RESULTS

Determination of the Optimal Concentration of Russell's Viper Venom.—A 1:40,000 dilution of Russell's viper venom in cephalin suspension was chosen as standard on the basis of the results summarized in Table I. They show the clotting times obtained with different dilutions of venom in 0.09 Gm. per cent cephalin. The shortest times were found in the 1:40,000 to 1:80,000 concentration range.

TABLE I. DETERMINATION OF OPTIMUM CONCENTRATION OF VENOM WHEN DISSOLVED IN 0.09 GM. PER CENT CEPHALIN SUSPENSION

CONCENTRATION OF VENOM DISSOLVED IN CEPHALIN SUSPENSION	CLOTTING TIME (SECONDS)
1:1,000	>600
1:5,000	45.3
1:10,000	32.0
1:20,000	28.6
1:40,000	28.0
1:80,000	28.0
1:160,000	33.4
1:320,000	41.1
1:640,000	51.5
1:1,280,000	65.5

Clotting mixture: 0.20 ml. ox plasma reagent, 0.20 ml. of various concentrations of venom dissolved in 0.09 Gm. per cent cephalin suspension, 0.20 ml. 1:50 dilution of standard plasma, 0.20 ml. 35 mM $CaCl_2$.

Determination of the Standard Concentration of Cephalin.—Table II gives the clotting times obtained when a 1:40,000 dilution of venom was made in varying concentrations of the cephalin suspension. A wide optimal concentra-

TABLE II. DETERMINATION OF THE OPTIMAL CEPHALIN SUSPENSION CONCENTRATION FOR VENOM CONCENTRATION OF 1:40,000

CONCENTRATION OF CEPHALIN SUSPENDED IN VERONAL BUFFER (GM. PER CENT)	CLOTTING TIME (SECONDS)
2.44	63.7
1.22	41.7
0.24	26.5
0.12	26.6
0.06	26.4
0.02	33.2
0.01	43.1
0.00	210 (?)

Clotting mixture: 0.20 ml. 1:40,000 venom dissolved in cephalin suspension of various concentrations. Otherwise as in Table I.

tion range for our particular cephalin preparation was found. A 1:30 dilution of the original 2.8 Gm. per cent suspension with buffer was selected as the standard procedure. This gives a cephalin suspension of 0.09 Gm. per cent.

Dissolving the venom in cephalin suspension eliminates variation in the clotting time due to the platelet or lipoid content of the test plasma. Table IIIA illustrates that platelet-rich (centrifuged 30 minutes at 900 r.p.m.) and platelet-poor plasmas (centrifuged 30 minutes at 2,500 r.p.m.) give the same

TABLE IIIA. COMPARISON OF PLATELET-RICH AND PLATELET-POOR SAMPLES OF THE SAME PLASMA

PLASMA	CLOTTING TIME (SECONDS)	
	PLATELET-RICH	PLATELET-POOR
1	26.4	26.4
2	26.3	25.9
3	24.0	24.3

TABLE IIIB. COMPARISON OF FASTING AND LIPEMIC PLASMA SAMPLES FROM THE SAME SUBJECT

PLASMA	PROTHROMBIN (PER CENT)	
	FASTING	LIPEMIC (90 MIN. AFTER DRINKING 330 ML. CREAM)
1	98	98
2	109	108
3	97	99

Clotting mixture: As in Table I, with Russell's viper venom 1:40,000 in 0.09 Gm. per cent cephalin suspension.

TABLE IV. DETERMINATION OF THE OPTIMUM CALCIUM CHLORIDE CONCENTRATION

CALCIUM CHLORIDE CONCENTRATION (mM)	CLOTTING TIME (SECONDS)
5	75.4
10	33.4
15	30.3
20	29.0
25	27.5
30	27.5
35	26.9
40	27.0
45	27.4
50	27.3
75	33.3
100	43.7

Clotting mixture: As in Table I, with Russell's viper venom 1:40,000 in 0.09 Gm. per cent cephalin suspension.

clotting times in this system. Table IIIB shows that lipemia produced by drinking cream does not alter the prothrombin values obtained by this technique.

The Optimal Calcium Chloride Concentration.—This was found, by the experiments summarized in Table IV, to lie between 25 and 50 mM. As cited above, a concentration of 35 mM has been chosen as standard.

Preparation of the Correlation Graph and Selection of the Dilution of the Test Plasma.—The prothrombin concentration of the test plasma is read from a correlation graph, such as Fig. 1, which relates the clotting time to the prothrombin content of dilutions of standard plasma. This graph is readily reproducible but requires meticulous care in making the dilutions. The times, when plotted on double logarithmic paper, are a straight line except for a bend at about 20 seconds. The reason for the bend is unknown. Above 20 seconds the line has the same slope as a thrombin-fibrinogen curve. The end point loses its sharpness above 80 seconds.

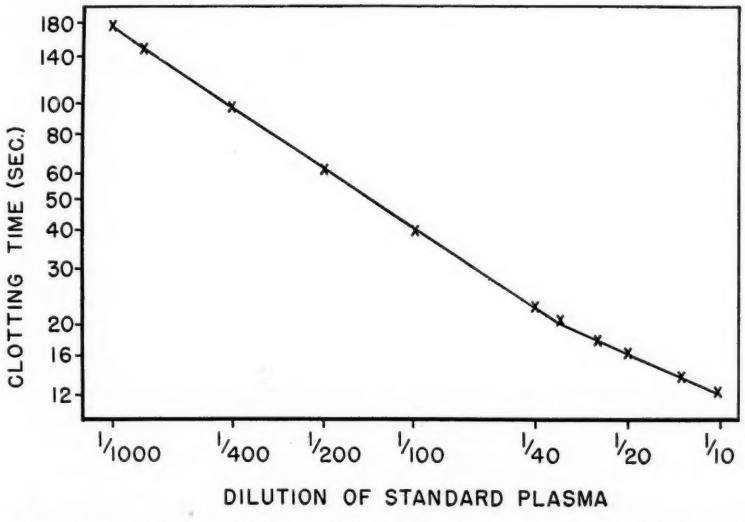

Fig. 1.—Correlation graph obtained by plotting the clotting times of dilutions of standard plasma on double logarithmic scale.

The optimum clotting times, therefore, are between 25 and 80 seconds. The clotting time will fall within this range for a 1:50 dilution of a test plasma with a prothrombin concentration between 20 and 120 per cent. (The clotting time of a 1:50 dilution of the standard plasma is arbitrarily called 100 per cent.) If the clotting time of a 1:50 dilution of the test plasma falls above or below this range, a 1:25 or a 1:100 dilution should be used.

Sensitivity of the Method to Heparin.—The P. and P. time is unaffected by small amounts of heparin in the test plasma or anticoagulant solution. However, heparin can lengthen the specific prothrombin time with venom-cephalin. Table V illustrates the increase in clotting time produced by a final concentration of about 1.4 μg of heparin (''A-L'', Oslo) in the dilutions of the test plasma. In these experiments, 100 μg of heparin were added to each

milliliter of the citrate anticoagulant. The first dilution to ten times the final concentration was made with Diluting Fluid I, which contained enough added heparin to keep the heparin concentration the same as in the undiluted citrated plasma sample. The final 1:10 dilution was made in the usual non-heparin-containing Diluting Fluid II.

TABLE V. THE EFFECT OF HEPARIN (1.4 μG PER MILLILITER OF DILUTED TEST PLASMA) UPON THE CLOTTING TIME

PLASMA DILUTION	CLOTTING TIME (SECONDS)	
	HEPARIN	NO HEPARIN
1:10	11.3	11.3
1:20	15.7	14.4
1:40	22.6	21.0
1:100	37.4	34.3
1:200	61.0	53.8
1:400	108.0	81.3

Clotting mixture: As in Table I, with Russell's viper venom 1:40,000 in 0.09 Gm. per cent cephalin suspension.

Absence of Variation Due to Glass Contact.—Tocantins[9, 10] first noted that the clot accelerating effect of glass contact is not found when Russell's viper venom is the thromboplastic agent. The reason is that glass contact increases the activity of both the antihemophilic B factor (PTC, Christmas factor) and proconvertin.[11] Venom plus cephalin acts like convertin and would therefore mask changes in the test plasma content of antihemophilic B factor or proconvertin.

TABLE VI. ABSENCE OF ACCELERATING EFFECT OF GLASS CONTACT UPON THE CLOTTING TIME

PLASMA	CLOTTING TIME (SECONDS)	
	SILICONE SAMPLE	GLASS-ACTIVATED SAMPLE
1	22.1	22.6
2	22.7	23.6
3	23.8	23.3
4	24.0	24.5
5	24.0	24.0

Clotting mixture: As in Table I, with Russell's viper venom 1:40,000 in 0.09 Gm. per cent cephalin suspension.

The data in Table VI verify that the specific prothrombin time with venom-cephalin does not vary with exposure of the test plasma to glass. Blood for these experiments was drawn with silicone technique (General Electric Dri Film 9987 for glassware and Arquard 2 C "Armour" for needles) and centrifuged for 15 minutes at 2,500 r.p.m. One portion of the plasma was shaken with quartz glass powder (Quartz gewaschen u. geglüht, E. Merck, Darmstad, ground in a glass mill to particles smaller than 0.068 mm.) for 10 minutes. A 1:50 dilution of this glass-activated plasma was then compared with a sample of the plasma exposed only to silicone surfaces. As can be seen, glass contact does not shorten the clotting time with venom-cephalin.

The insensitivity of the method to the effects of glass contact and the stability of prothrombin permits storage of the plasma for several days with-

TABLE VII. THE EFFECT OF STORAGE UPON THE PROTHROMBIN CONCENTRATION OF NORMAL PLASMA

STORAGE TIME (DAYS)	PROTHROMBIN (PER CENT)			
	PLASMA 1	PLASMA 2	PLASMA 3	PLASMA 4
0	100	97	91	107
1	98	96	87	107
2	97	96	90	98
4	99	92	86	98
6	101	97	86	96
8	95	96	85	100
10	83	79	73	86
14	81	75	74	84

Clotting mixture: As in Table I, with Russell's viper venom 1:40,000 in 0.09 Gm. per cent cephalin suspension.

out influencing the result of the test. This is illustrated by the data in Table VII which show that the plasma may stand under sterile conditions for as long as eight days at room temperature without introducing an error.

Concentration of Prothrombin in Normal and Pathologic Plasmas.—When the arbitrarily selected standard plasma was called 100 per cent, the average value for the plasma prothrombin content of 35 normal subjects was 105 per cent. The range was 92 to 120 per cent. As expected, normal values for prothrombin concentration were found in patients with hemophilia A and B, parahemophilia, and congenital hypoproconvertinemia (Table VIII). The normal values in the patients with congenital hypoproconvertinemia again confirms the independence of the clotting action of venom-cephalin and proconvertin.

TABLE VIII. THE PROTHROMBIN CONCENTRATION IN VARIOUS PATHOLOGIC PLASMAS

DIAGNOSIS	PROTHROMBIN (PER CENT)
1. Hemophilia A	93
2. Hemophilia B	105
3. Hemophilia B	108
4. Hemophilia B	126
5. Hemophilia B	103
6. Congenital hypoproconvertinemia	122
7. Congenital hypoproconvertinemia	113
8. Congenital hypoproconvertinemia	108
9. Parahemophilia	120

DISCUSSION

The venom-cephalin test is a simple one-stage clotting test sensitive only to variation in the prothrombin content of the test plasma. This is so because the venom-cephalin reagent supplies the clotting equivalent of an optimum amount of convertin, while the high proaccelerin and fibrinogen content of the ox plasma protects against changes in the test plasma content of these factors. The ox plasma also helps to stabilize the antithrombin titer of the system. A 1:50 dilution of the test plasma will permit a sensitive and accurate measurement of a prothrombin concentration between 20 and 120 per cent of normal.

The reagents are simple to prepare. The viper venom is readily available commercially.* A substitute for cephalin is also available.† However, the cephalin and ox plasma reagents are easily prepared in the ordinary laboratory. The anticoagulant solution should not contain heparin as recommended for some brain thromboplastin methods.[12] Small amounts of heparin can increase the clotting time with venom-cephalin. Recent administration of heparin to the patient could also alter the results.

Neither this nor any other test which measures prothrombin alone should be used to control anticoagulant therapy with Dicumarol or similar drugs. These drugs produce a proconvertin deficit that can lead to bleeding without a striking depression of prothrombin. Obviously, this proconvertin depression will be missed by a test which is specific for prothrombin. A test which utilizes brain thromboplastin, such as the P. and P. test, must be used to control Dicumarol therapy.

The venom-cephalin test should prove valuable to those who use prothrombin consumption tests. One of the difficulties with the prothrombin consumption test is that the prolongation of the serum prothrombin time determined with brain thromboplastin is partially masked by the formation of convertin and active proconvertin during clotting.[11] This source of error is eliminated with the venom-cephalin method. Venom-cephalin should also prove useful as a substitute for tissue thromboplastin in the usual two-stage prothrombin methods. The reduced converting time with venom-cephalin will diminish the proportion of thrombin inactivated by antithrombin in the incubation mixture.

TABLE IX. DIFFERENTIATION OF COAGULATION DEFECTS NOT CONCERNED WITH THROMBOPLASTIN FORMATION

COAGULATION FACTOR INSUFFICIENT	PROLONGED CLOTTING TIME		
	QUICK TEST	P. AND P. TEST	VENOM-CEPHALIN
Prothrombin	Yes	Yes	Yes
Proconvertin	Yes	Yes	No
Proaccelerin	Yes	No	No
Fibrinogen	Yes	No	No

The use of the Quick test, the P. and P. test, and the venom-cephalin prothrombin test provides a rapid and simple way to differentiate clotting defects caused by deficiencies of prothrombin, proconvertin, proaccelerin, and fibrinogen. The findings in each of these deficiencies are given in Table IX.

SUMMARY

A simple one-stage test for prothrombin is described. The principal reagents are (1) Russell's viper venom dissolved in cephalin suspension, and (2) adsorbed ox plasma. The method is sensitive and specific. It is uninfluenced by variation in the test plasma content of lipoid, platelets, plasma thromboplastin precursors, proconvertin, proaccelerin, or fibrinogen. Neither this nor

*Stypven, Burroughs Wellcome & Co., Inc.
†Asolectin, Associated Concentrates, Inc., New York.

any method which measures prothrombin alone should be used to control Dicumarol therapy, for the proconvertin deficiency which this drug also produces will not be measured.

We wish to thank Nurse Solveig Mikkelsen for her help in these experiments.

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HEREDITARY DEFICIENCY OF PROACCELERIN (PARAHEMOPHILIA): A FAMILY STUDY

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IT IS now recognized that the conversion of prothrombin to thrombin by tissue (brain) thromboplastin requires the participation of two "accessory" factors. One, a labile globulin termed proaccelerin by Owren, functions as an accelerator of prothrombin conversion. The other, a stable component of plasma which persists in serum after clotting, has been termed proconvertin[1] to indicate its basic action in the conversion of prothrombin to thrombin (Table I). Since a deficiency or absence of either of these factors will result in a prolonged "prothrombin time" it is no longer accurate to regard such a finding as synonymous with hypoprothrombinemia.

TABLE I. SYNONYMS OF PROACCELERIN AND PROCONVERTIN

PROACCELERIN (OWREN, 1952)	PROCONVERTIN (OWREN, 1952)
Prothrombin A (Quick, 1943)	Serum prothrombin conversion factor (SPCA) (Alexander, 1949, 1950, 1951, 1952)
Prothrombin Accelerator (Fantl and Nance, 1946)	Co-thromboplastin (Mann, 1949, 1950, 1951)
Factor V (Owren, 1947)	Stable prothrombin conversion factor (Owren, 1948, 1951)
Labile Factor (Quick, 1947)	Factor VII (Koller, 1951, 1952)
Plasma accelerator Globulin (Ware et al., 1947)	
Plasmatic co-factor of thromboplastin (Honorato, 1947)	
Plasma prothrombin conversion factor (Stefanini, 1951)	

In 1947 Owren[2, 3] described the case of a 29-year-old woman who gave a history of abnormal bleeding since the age of 3½ years. Investigation of this patient disclosed prolongation of the whole blood clotting time as well as a prolonged "prothrombin time," both of which could be corrected by the addition of prothrombin-free fresh normal plasma. She was considered to have a congenital deficiency of the labile globulin substance which Owren called, at that time, Factor V. He suggested the name "parahemophilia" for this disorder. Similar cases have since been described by a number of investigators (Table II). Although the disease occurs in a familial fashion, the manner of hereditary transmission has been obscure. Most previous reports have either failed to include studies on the parents or, if done, they were of such a limited nature as to reveal no consistent abnormalities (Table II). The pur-

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TABLE II. REPORTED CASES OF PARAHEMOPHILIA

CASES	SEX	AGE (YR.)	PARENTS AFFECTED	SIBLINGS AFFECTED	REMARKS
Owren[2, 3]	Female	29	No	No	Original case
De Vries et al.[4]	Female	11	No	Four other siblings unaffected. One with syndactylism	Syndactylism
	Male	7			
	Male	15			
Alexander and Goldstein[5]	Female	17	No	—	
	Female	15			
	Male	4			
Frank et al.[6]	Male				
Stohlman et al.[7]	Female		No	No	
Brink and Kingsley[8]	Female	27	No	Seven unaffected siblings	Abnormal bleeding in both paternal and maternal ancestors
	Female	32			
	Male	13			
Brennan et al.[10]	Male	3	No	Three unaffected	
Authors'	Male	2½	No	—	
	Male	7			

pose of this paper is to present two new cases of parahemophilia in siblings, with studies on their parents which may provide a clue to the genetic nature of the disease.

CASE REPORTS

The patients were brothers, T. H., aged 7 years, and S. H., aged 2½ years, the only children of a couple of Irish-American ancestry. The parents, highly intelligent persons, could give no familial history of a hemorrhagic tendency nor were they themselves affected by any unusual bleeding. There was no consanguinity. T. H. was circumcised at the age of 7 weeks and had an alarming hemorrhage which necessitated a transfusion. Since then he had been subject to spontaneous epistaxes some of which had been quite profuse. He had also developed subcutaneous hematomas and ecchymoses with minor trauma on numerous occasions and at various times had required hospitalization and transfusions. S. H. bled profusely for the first time at the age of 8 months, following a laceration of the lip. He also had had epistaxes and other hemorrhagic manifestations requiring transfusion. Neither brother had had any episodes of hemarthrosis. It had been noted in the past that transfusion of fresh whole blood from the father had a good hemostatic effect. The remainder of the history was essentially negative. Physical examination was entirely normal except for the presence of scattered ecchymoses on the lower extremities of both children.

During the preparation of this paper for publication, S. H. fell a distance of several feet from a toy wagon and struck his head on a cement surface. Several hours later he awakened from a nap, vomited, and had several generalized convulsions. He was admitted to the hospital, transfused, and a craniotomy was performed. A large subdural hematoma was found which was evacuated. The operator noted numerous petechial hemorrhages on the surface of the brain. The patient failed to regain consciousness and died twelve hours later. Autopsy revealed a large accumulation of intracerebral blood in addition to the remnants of the subdural hemorrhage.

Initial laboratory studies revealed the following: T. H.: Hemoglobin, 11.6 Gm.; red blood cells, 4.9 million per cubic millimeter; white cell count and differential normal; platelet count, 833,000 (Olef); bleeding time, 5½ minutes; coagulation time (Lee-White),

28 minutes, 48 minutes, and 58 minutes, in the first, second, and third tubes, respectively; capillary fragility test negative; "prothrombin time" (Quick), 110 seconds (less than 5 per cent). S. H.: Hemoglobin, 12.7 Gm.; red blood cells, 4.69 million per cubic millimeter; white blood cell count and differential count normal; platelet count, 1,085,000; bleeding time, 3 minutes; coagulation time, 40 minutes, 49 minutes, and 57 minutes in the first, second, and third tubes, respectively; capillary fragility test negative; "prothrombin time" (Quick), 102 seconds (less than 5 per cent).

Bleeding and clotting times were perfectly normal in the father and mother. The former's prothrombin time was 15 seconds (100 per cent) and the latter's also 15 seconds (100 per cent).

METHODS AND MATERIALS

Silicone-coated syringes and glassware were used for collection of blood samples unless otherwise required by a specific method. Anticoagulant solutions employed were 1.34 per cent and 2.0 per cent potassium oxalate solutions, and 3.8 per cent sodium citrate. These were mixed with blood in the proportion of 1:10. Bleeding time was determined by the method of Duke,[11] clotting time by the Lee-White[12] procedure. "Prothrombin time" was determined by the one-stage method of Quick[13] and the P and P method of Owren and Aas,[14] as well as by the two-stage methods of Warner, Brinkhous, and Smith[15] and Owren.[2] Prothrombin consumption was studied by Quick's method.[16] Quantitative proaccelerin determinations were made by the method of Wolf.[17]

Commercial thromboplastin (Difco) and thromboplastin prepared from aqueous extracts of human brain were used. Bovine thrombin (Upjohn) was employed when needed. Fibrinogen in the form of bovine plasma fraction I (Armour), or prepared by the method of Jaques,[33] was employed. Plasma free of prothrombin and proconvertin (Factor VII) was prepared by the method of Owren and Aas[14] using filter pads containing 20 per cent and 50 per cent asbestos (Hercules Filter Company: C-5 = 20 per cent, ST-3 = 50 per cent). Similar plasma was also prepared by barium sulfate[5, 18] or aluminum hydroxide (added in proportion of 5 per cent) absorption.[19, 20] Plasma devoid of proaccelerin was prepared either by the method of Wolf[17] or by the use of aged plasma. Normal human plasma and serum, both fresh and aged, were used where indicated. Additional comments on some of these reagents are made below.

PATIENT STUDIES

1. *One-Stage "Prothrombin Time"* (Quick).—Tests were carried out in the usual manner in nonsilicone-coated tubes. Difco thromboplastin was employed. Tests done on three different occasions (Table III) gave values no less than 105 seconds with T. H.'s plasma nor less than 95 seconds with S. H.'s plasma. The end point was often difficult to detect because of the slow formation of fibrin and the spongy quality of the clot. An interesting phenomenon was observed when the tests were carried out at varying intervals after the collection of the blood. It will be noted (Table III) that there was distinct shortening of the "prothrombin time" in those tests performed 90 to 180 minutes after blood collection. This observation is discussed further below.

2. *Prothrombin Consumption Test* (Quick).—Serum prothrombin activity was elevated in both patients. One hour after coagulation, values of 50 per cent and 65 per cent were obtained with T. H.'s and S. H.'s serum, respectively.

3. *Two-Stage Prothrombin Time* (Warner, Brinkhous, and Smith, 1936; Owren, 1947).—With the former method (without proaccelerin supplement), delayed formation of thrombin (no clotting of fibrinogen within the first six minutes) could be demonstrated in both patients. The possibility that this might be due to the action of an antithrombin was excluded by the fact that the addition of thrombin (0.1 ml. of a dilution containing 10 units per milliliter) produced clotting of the patients' plasma in 10-12 seconds. Furthermore, the ability of normal plasma to correct the clotting defect likewise ruled out antithrombic

TABLE III. BEHAVIOR OF ONE-STAGE "PROTHROMBIN TIME" AT DIFFERENT INTERVALS AFTER COLLECTION OF BLOOD

TIME ELAPSED AFTER COLLECTION OF BLOOD (MINUTES)	AUG. 8, 1953		AUG. 19, 1953		SEPT. 5, 1953	
	T. H.	S. H.	T. H.	S. H.	T. H.	S. H.
15	>10′		>10′	160″	256″	120″
30	>10′			165″		
45				97″	173″	
60			200″	99″	112″	98″
90	126″		157″	98″	105″	95″
120	118″		110″	95″	109″	95″
180	120″		110″	104″	108″	

activity in the patient's plasma (Table V). In Owren's modification of the two-stage prothrombin test proaccelerin is added in the form of filtered beef plasma. With this technique, normal formation of thrombin was observed.

4. *The Effect of Hemophilic Plasma.*—The calcium clotting time of the hemophilic plasma used was 13 minutes, while that of T. H.'s plasma alone was greater than 15 minutes. Mixture of the two (1:1 and 9:1) shortened the calcium clotting time to 118 seconds. Hemophilic plasma was somewhat more effective in correcting the one-stage "prothrombin time" than normal plasma (Fig. 1).

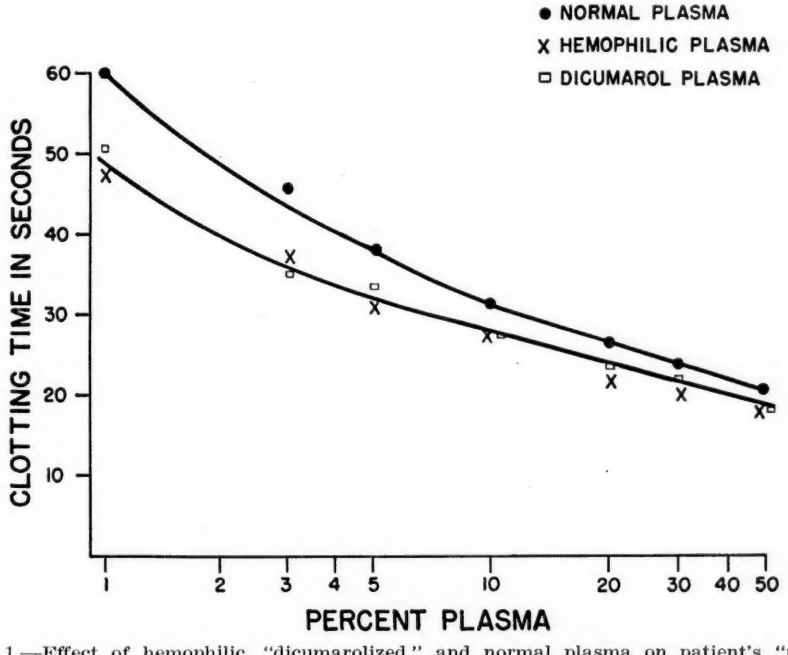

Fig. 1.—Effect of hemophilic, "dicumarolized," and normal plasma on patient's "prothrombin time." (Per cent plasma refers to amount of test plasma added to patient's plasma.)

5. *Effect of Prothrombin and Proconvertin Free Plasma.*—Plasma devoid of both prothrombin and proconvertin and of each individually was prepared in various ways. Treatment with $A_1(OH)_3$ or $BaSO_4$ removes both constituents. Filtration through filter pads containing 20 per cent asbestos selectively removes proconvertin while filtration through 50 per cent asbestos removes both prothrombin and proconvertin. Mixture of these reagent plasmas with patient plasma showed uniform correction of the prolonged "prothrombin time" (Table IV, Figs. 2, 3, and 4).

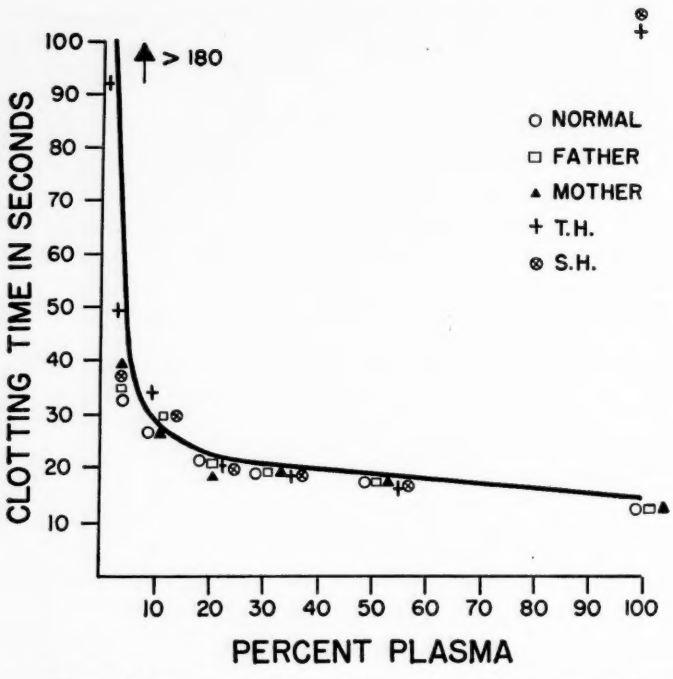

Fig. 2.—Effect of normal, patient's, and parents' plasma on prothrombin and proconvertin-free beef plasma. (Per cent plasma refers to amount of test plasma added to beef plasma.)

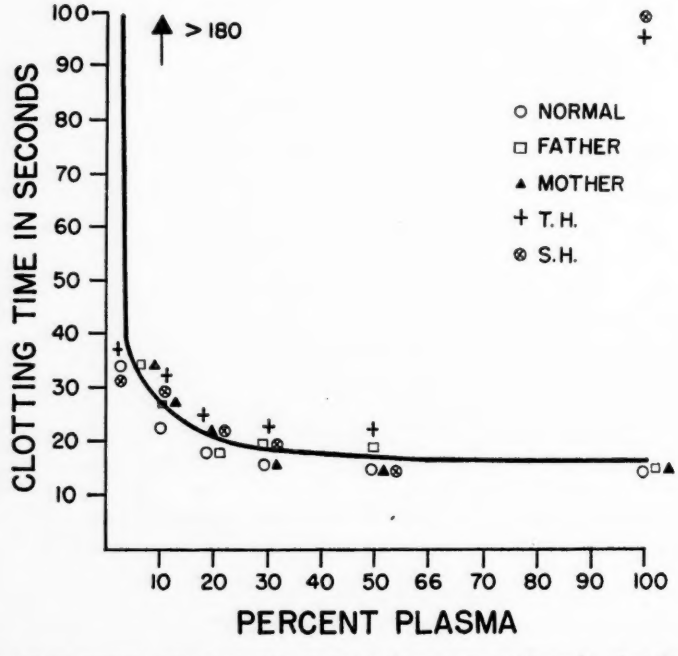

Fig. 3.—Effect of normal, patient's, and parents' plasma on prothrombin-free beef plasma. (Per cent plasma refers to amount of test plasma added to beef plasma.)

TABLE IV. EFFECT OF PLASMA FREE OF PROTHROMBIN AND PROCONVERTIN ON THE ONE-STAGE "PROTHROMBIN TIME" OF PATIENTS' PLASMA

T. H. PLASMA (2 HR. OLD)	S. H. PLASMA (2 HR. OLD)	NORMAL PLASMA	$Al(OH)_3$-TREATED PLASMA	$BaSO_4$-TREATED PLASMA (100 MG.)	ONE STAGE "PROTHROMBIN TIME" (SEC.)
Parts					
1	-	-	-	-	105
-	1	-	-	-	95
1	9	-	-	-	100
9	1	-	-	-	98
-	-	1	-	-	16
9	-	1	-	-	19
-	9	1	-	-	22
-	-	-	1	-	>180
9	-	-	1	-	28
-	9	-	1	-	32
-	-	-	-	1	>180
9	-	-	-	1	25
-	9	-	-	1	23

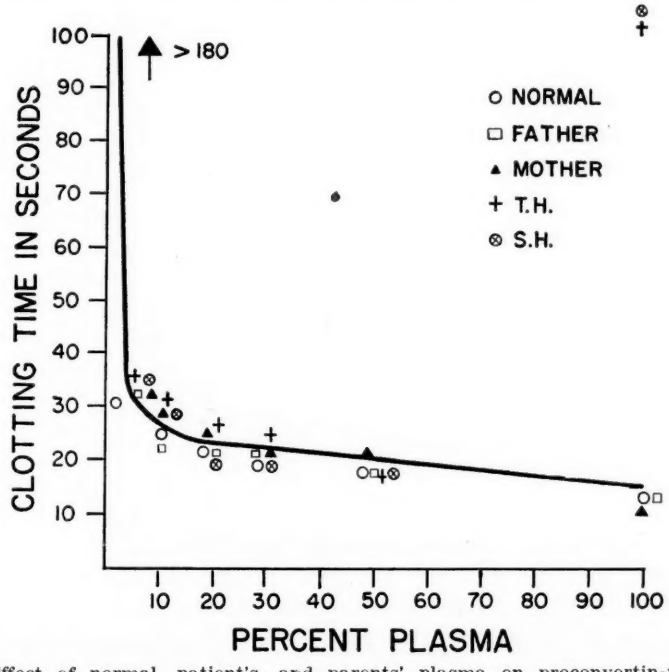

Fig. 4.—Effect of normal, patient's, and parents' plasma on proconvertin-free beef plasma. (Per cent plasma refers to amount of test plasma added to beef plasma.)

6. *Effect of Proaccelerin-Free Plasma on One-Stage "Prothrombin Time".*—Proaccelerin activity disappears from plasma stored 15 to 25 days at 4° C. Wolf has recently reported that normal plasma incubated for 24 hours at 37° C. likewise loses its proaccelerin activity.[17] The addition of both aged and incubated plasma failed to correct the "prothrombin time" of the patient's plasma. Mixture of the brothers' plasma likewise showed no mutual corrective effect. When studied by Wolf's modification[17] of Stefanini's method,[21-23] the proaccelerin content of the patients' plasma was found to be less than 1 per cent.

7. *Influence of Serum.*—Proaccelerin is present in small amounts in normal fresh serum but is said to disappear within a few hours.[24, 25] Experiments were carried out to determine the influence of normal sera, stored for varying periods at different temperatures, on

our patients' "prothrombin time." Fresh normal serum was found to have a corrective effect. Sera stored at 6° C. continued to show some proaccelerin activity up to approximately 15 days. In serum stored at room temperature (28° to 37° C. during the experiments) virtually no proaccelerin activity was present after 3 days. Fresh oxalated serum treated with $Al(OH)_3$ or $BaSO_4$ had no corrective effect. (Table V.)

TABLE V. INFLUENCE OF PATIENT'S SERUM, STORED SERUM, $Al(OH)_3$-, AND $BaSO_4$-TREATED SERA ON THE ONE-STAGE "PROTHROMBIN TIME"

T. H. PLASMA	T. H. FRESH SERUM	S. H. PLASMA	S. H. FRESH SERUM	NORMAL FRESH SERUM	NORMAL SERUM AFTER 3 DAYS AT 6° C.	NORMAL SERUM AFTER 7 DAYS AT 6° C.	NORMAL SERUM AFTER 20 DAYS AT ROOM TEMP.	NORMAL FRESH OXALATED "ALUMINA" SERUM	NORMAL FRESH OXALATED $BaSO_4$-TREATED SERUM	TIME (SEC.)
1	–	–	–	–	–	–	–	–	–	114
–	–	1	–	–	–	–	–	–	–	98
9	–	–	1	–	–	–	–	–	–	109
–	1	9	–	–	–	–	–	–	–	101
9	–	–	–	1	–	–	–	–	–	31
–	–	9	–	–	1	–	–	–	–	42
9	–	–	–	–	–	1	–	–	–	58
–	–	9	–	–	–	–	1	–	–	81
9	–	–	–	–	–	–	–	1	–	113
9	–	–	–	–	–	–	–	–	1	100

The lability of proaccelerin must be carefully defined in view of these data. Since certain types of stored serum could restore the "prothrombin time" of our patients' plasma, one might be tempted to postulate that a double deficiency, i.e., proaccelerin and proconvertin, existed. That a deficiency of proconvertin did not exist was demonstrated by (1) the normal values obtained with the P and P test of Owren; (2) the ability of the patients' plasma to correct the "prothrombin time" of "dicumarolized" plasma; (3) the ability of the patients' plasma to correct the defect of $BaSO_4$-treated plasma; (4) normal values obtained with the modified two-stage prothrombin test which is supplemented by the addition of proaccelerin. These tests incidentally also ruled out a coexisting prothrombin deficiency which, according to Alexander, may sometimes occur in parahemophilia.[5] From the data cited above it seems likely that $Al(OH)_3$ and $BaSO_4$ have a minor adsorptive effect on proaccelerin as well as on prothrombin and proconvertin. Since plasma contains a relative abundance of proaccelerin, the slight decrease passes unnoticed, but the effect is apparent in serum because of the paucity of the material.

8. *Effect of Plasma and Sera of Patients Under Treatment With Dicumarol or Tromexan on Parahemophilic Plasma.*—The ability of plasma and serum from patients under treatment with dicumarol or tromexan to restore the "prothrombin time" of parahemophilic plasma was tested. Such plasma was found to correct the patients' "prothrombin time" even somewhat more efficiently than normal plasma. (Table VI, Fig. 1.) Sera from these patients were also somewhat more active than normal sera. The proaccelerin activity of "dicumarolized" plasma and serum was less affected by storage than normal plasma or serum. It was observed that treatment with $BaSO_4$, which completely removes the proaccelerin activity of normal sera, affected the "dicumarolized" serum to a lesser degree.

STUDIES ON PARENTS' BLOOD

As previously stated, routine investigation failed to reveal any abnormality of the parents' blood. Owren's P and P test was also normal. The ability of their plasma equaled that of normal plasma (Table VII) in correcting the coagulation defect of the

TABLE VI. INFLUENCE OF DICUMAROL AND TROMEXAN PLASMA AND SERUM ON PARAHEMOPHILIC PLASMA

T. H. PLASMA	S. H. PLASMA	OXALATED DICUMAROL PLASMA (J. S.)	PLASMA AFTER $Al(OH)_3$ TREATMENT (J. S.)	DICUMAROL FRESH SERUM (J. S.)	TROMEXAN OXALATED PLASMA (B. L.)	TROMEXAN OXALATED $Al(OH)_3$-TREATED PLASMA	TROMEXAN OXALATED $BaSO_4$-TREATED PLASMA	TROMEXAN SERUM	TIME
1	-	-	-	-	-	-	-	-	110
-	1	-	-	-	-	-	-	-	107
-	-	1	-	-	-	-	-	-	36
-	-	-	1	-	-	-	-	-	>180
-	-	-	-	-	1	-	-	-	38
-	-	-	-	-	-	1	-	-	>180
-	-	-	-	-	-	-	1	-	>180
9	-	1	-	-	-	-	-	-	23
-	9	-	1	-	-	-	-	-	50
-	9	-	-	1	-	-	-	-	25
9	-	-	-	-	1	-	-	-	27
-	9	-	-	-	1	-	-	-	27
9	-	-	-	-	-	1	-	-	35
9	-	-	-	-	-	-	1	-	29
9	-	-	-	-	-	-	-	1	26

children's plasma. An important difference was found when the corrective effect of their sera was compared with that of normal serum. It will be observed (Table VII) that the addition of fresh normal serum reduced the "prothrombin time" of S. H.'s plasma from 96 to 35 seconds. On the other hand, the addition of fresh maternal or paternal serum reduced the time only to 60 seconds. A deficit in the proaccelerin content of the parents' plasma was suspected.

It is generally agreed that proaccelerin exists in an inactive form (plasma AC-globulin, etc.) in plasma, but is transformed into an active form (accelerin, serum AC-globulin) during coagulation. Fresh serum obtained by the treatment of normal plasma with thrombin[5] (2.0 units in 0.10 ml. of saline per milliliter of plasma) had a marked restorative effect on the "prothrombin time" of aged (28-day-old) plasma and incubated plasma (Table VIII). T. H.'s and S. H.'s plasma treated in a similar manner had virtually no corrective effect. Maternal and paternal sera prepared in the same way displayed a distinct deficit in restorative effect as compared to normal sera. In these experiments 0.1 ml. of serum was added to 0.4 ml. of aged plasma. Expressed quantitatively normal serum obtained from plasma treated with thrombin had an accelerin value of over 100 per cent, whereas the father's serum had only 10 per cent. The parents' sera tested one-half hour after natural coagulation of whole blood gave values of less than 1 per cent, whereas normal sera had an accelerin content equivalent to 10 per cent (60 seconds with both parents' sera, 35 seconds with normal sera).

Reference to Figs. 2, 3, and 4 reveals that a considerable reduction of the various factors of the plasma must be present before significant alterations in the one-stage procedure will be detected. The normality of routine test procedures plus the lack of clinical symptomatology probably account for the previous failure to establish a firm genetic basis for this disease.

DISCUSSION

Hereditary deficiency of proaccelerin occurs in both males and females (Table II). A hemorrhagic tendency is noted early in life and is characterized by excessive bleeding from minor or major traumas as well as by occasional episodes of "spontaneous" bleeding. Unlike hemophilia, hemarthroses are uncommon. Aside from the occasional finding of an associated anemia the only abnormalities found on routine laboratory study are prolonged co-

TABLE VII. EFFECT OF NORMAL, MATERNAL, AND PATERNAL PLASMA AND SERUM ON PATIENTS' "PROTHROMBIN TIME"

		NORMAL		MATERNAL					PATERNAL						
T. H. PLASMA	S. H. PLASMA	PLASMA	FRESH SERUM	PLASMA	$BaSO_4$-TREATED PLASMA	FRESH SERUM	OXAL. "ALUMINA" SERUM	OXAL. $BaSO_4$-TREATED SERUM	PLASMA	"ALUMINA" PLASMA	$BaSO_4$-TREATED PLASMA	FRESH SERUM	OXAL. "ALUMINA" SERUM	OXAL. $BaSO_4$-TREATED SERUM	TIME (SEC.)
1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	107
–	1	–	–	–	–	–	–	–	–	–	–	–	–	–	96
–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	15
–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	15
–	–	–	–	–	1	–	–	–	–	–	–	–	–	–	>180
–	–	–	–	–	–	–	–	–	1	–	–	–	–	–	16
–	–	–	–	–	–	–	–	–	–	1	–	–	–	–	>180
–	–	–	–	–	–	–	–	–	–	–	1	–	–	–	>180
9	–	1	–	–	–	–	–	–	–	–	–	–	–	–	18
–	9	1	–	–	–	–	–	–	–	–	–	–	–	–	21
–	9	–	1	–	–	–	–	–	–	–	–	–	–	–	35
–	9	–	–	1	–	–	–	–	–	–	–	–	–	–	18
–	9	–	–	–	1	–	–	–	–	–	–	–	–	–	29
9	–	–	–	–	1	–	–	–	–	–	–	–	–	–	32
–	9	–	–	–	–	1	–	–	–	–	–	–	–	–	60
–	9	–	–	–	–	–	1	–	–	–	–	–	–	–	97
–	9	–	–	–	–	–	–	1	–	–	–	–	–	–	101
–	9	–	–	–	–	–	–	–	1	–	–	–	–	–	20
9	–	–	–	–	–	–	–	–	1	–	–	–	–	–	19
–	9	–	–	–	–	–	–	–	–	1	–	–	–	–	42
–	9	–	–	–	–	–	–	–	–	–	1	–	–	–	34
–	9	–	–	–	–	–	–	–	–	–	–	1	–	–	60
–	9	–	–	–	–	–	–	–	–	–	–	–	1	–	99
–	9	–	–	–	–	–	–	–	–	–	–	–	–	1	102

TABLE VIII. RESTORATIVE EFFECT OF THROMBIN-TREATED PLASMAS ON PROACCELERIN-FREE PLASMA

	"PROTHROMBIN TIME" (SEC.)	"PROTHROMBIN TIME" (SEC.) AFTER INCUBATION		
		15′	45′	90′
Aged Plasma and Saline	112	-	-	-
Aged Plasma and Fresh Normal Plasma	23	-	-	-
Normal Plasma Incubated 24 hours at 37° C. plus Saline Solution	86	-	-	-
Incubated Plasma and Fresh Normal Plasma	17	-	-	-
Aged Plasma plus				
Thrombin-Treated Normal Plasma		17	17	19
Thrombin-Treated T. H. Plasma		90	92	97
Thrombin-Treated S. H. Plasma		88	85	93
Thrombin-Treated Mother's Plasma		37	35	41
Thrombin-Treated Father's Plasma		29	31	31
Incubated Plasma plus				
Thrombin-Treated Normal Plasma		16	15	18
Thrombin-Treated T. H. Plasma		93	91	99
Thrombin-Treated S. H. Plasma		96	96	102
Thrombin-Treated Mother's Plasma		37	37	43
Thrombin-Treated Father's Plasma		25	28	29

agulation time and prolonged "prothrombin time." The latter finding excludes the diagnosis of hemophilia as well as the recently described hemophilia-like syndromes (Christmas disease, etc.) but does not exclude fibrinogenopenia, circulating anticoagulants, hypoprothrombinemia, and hypoproconvertinemia (Factor VII, SPCA deficiency). Fibrinogen deficiency is readily excluded by direct chemical quantitation of this substance in the plasma. The presence of coagulation inhibitors may be excluded if the addition of patient's plasma to normal plasma fails to produce a prolongation of the recalcification time or "prothrombin time."

Prior to the discovery of the prothrombin accessory factors a number of reports of idiopathic hypoprothrombinemia appeared in the literature. It seems likely that many of these were actually congenital proaccelerin (Factor V) or proconvertin deficiencies.[26] The differentiation of these syndromes can be accomplished very simply. Plasma devoid of prothrombin and proconvertin can be prepared by absorption with barium sulfate or aluminum hydroxide solution or by filtration through pads containing asbestos. The addition of such plasma to the patient's plasma with resultant correction of the "prothrombin time" strongly suggests a defect based on proaccelerin deficiency. On the other hand, the correction of the patient's defect by the addition of stored normal serum would indicate proconvertin deficiency.

Although the familial occurrence of proaccelerin deficiency has been noted,[4, 32] the exact mode of inheritance has not been clear. It seems likely that the failure to establish a definite genetic pattern in a number of the previously reported cases was due largely to the inadequacy of the testing procedures in so-called "normal" members of the family group. The parents of our patients had normal "prothrombin times." When the residual proaccelerin of their fresh serum, after spontaneous coagulation or after treatment of their plasma with thrombin, was determined, a marked deficiency

was found. On the basis of these data the following genetic hypothesis seems likely. The mutant gene responsible for proaccelerin deficiency may exist in either homozygous or heterozygous state. In the latter situation a subclinical deficiency exists which is not characterized by a hemorrhagic diathesis. In its homozygous state the frank disease, parahemophilia, occurs. In a recent study, Kingsley[9] assayed the proaccelerin content of the blood in two families some of whose members were clinically normal while others had parahemophilia. He also concluded that partial deficiency of proaccelerin occurred in carriers who were probably heterozygous for the abnormal gene. Additional family studies will obviously be necessary to validate the hypothesis mentioned above. The incidence of partial deficiency of proaccelerin in large population groups would seem to be a problem worthy of further study.

In the course of our study certain observations regarding the proaccelerin content of blood were made. Although this factor is labile, the concept must be carefully qualified. Thus sera stored at room temperature for less than 3 days or at 6° C. for less than 10 to 15 days were still found to contain enough proaccelerin to give false results in studies requiring aged plasma. We were also able to confirm the fact that incubation for 24 hours at 37° C., as suggested by Wolf,[17] results in a proaccelerin-free reagent which may be used in place of aged plasma. It was interesting to note that the plasmas of "dicumarolized" patients, patients treated with tromexan, and hemophiliacs seem to have an increased proaccelerin activity. Similar observations were made by Alexander and Goldstein[5] in parahemophilia and by Stefanini and Crosby[27] in thrombocytopenias as well as in the presence of inhibitors such as heparin, i.e., in situations in which the evolution of thrombin is, in general, retarded.

Acceleration of the "prothrombin time" of parahemophilic plasma tested at varying intervals after venesection is a phenomenon requiring further study. Ware and associates[28] in 1948 and McClaughry and Seegers[29] in 1950 isolated a substance from platelets which had an action similar to proaccelerin. It is interesting to consider the possibility that shortening of the "prothrombin time" might be due to the slow liberation of such a substance from platelets. A somewhat analogous phenomenon observed in hemophilia, i.e., shortening of the calcium clotting time after plasma has been kept for two hours at room temperature, has been ascribed to slow lysis of platelets in this disease. In general the slow evolution of thrombin, whenever present, apparently retards the lysis of platelets.[30, 31]

The differentiation of proaccelerin deficiency from hypoprothrombinemia and/or hypoproconvertinemia is of more than academic interest. Although the temptation to treat patients with prolonged "prothrombin time" with synthetic vitamin K or vitamin K_1 oxide is great, there is no evidence that such therapy has any influence on proaccelerin deficiency. Transfusion with fresh whole blood thus far represents the best method of management of parahemophilia.

SUMMARY

1. Two brothers, aged 2½ and 7 years, respectively, with a hereditary hemorrhagic diathesis characterized by a prolonged coagulation time and a prolonged "prothrombin time" were studied and found to have a deficiency of proaccelerin (Factor V, labile factor).

2. Study of the parents revealed normal values for coagulation time and "prothrombin time." However, study of the residual proaccelerin in their fresh sera revealed subclinical deficiency of proaccelerin.

3. A genetic hypothesis for parahemophilia (hereditary deficiency of proaccelerin) is proposed. The carrier state, exemplified by the parents, is the result of the presence of the mutant gene in heterozygous form, whereas the frank disease, exemplified by the children, represents the mutant gene in homozygous form.

ADDENDUM

Since the submission of this paper for publication a third child has been born. Tests reveal a normal amount of proaccelerin. This finding further validates the hypothesis of a heterozygous state in the parents.

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STUDIES ON THE STORAGE LABILITY OF HUMAN ANTI-HEMOPHILIC FACTOR

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TRADITIONALLY, human antihemophilic factor (AHF) has been considered a storage-labile protein. This viewpoint has been reaffirmed in several recent publications.[1-3] However, Alexander[4] reported only 50 per cent loss of AHF in refrigerated plasma stored for five weeks, and Brinkhous[5] has recently stated that AHF is less storage labile than has been supposed formerly. Two possible explanations for these divergent findings are the suggestion by Biggs and Macfarlane[6] that there may be considerable individual variability in AHF lability, and the findings of Penick and Brinkhous[7] which indicate that AHF is more stable at cold temperatures, in nonwetting containers, and in citrated plasma. If these factors are not controlled, discrepancies in the estimation of AHF lability are bound to occur.

In the present study it will be shown that human AHF is highly labile if oxalate is used as the anticoagulant, whereas it is considerably more stable in citrated plasma. By way of contrast, bovine AHF has been shown to be highly storage-stable in oxalated plasma.[3] Advantage has been taken of this difference between human and bovine plasma to elucidate the mechanism of the storage lability of human AHF in oxalated plasma. It was reasoned that one of three possible factors was responsible for the increased stability of bovine AHF: (1) bovine plasma has some protective factor which preserves AHF; (2) human and bovine AHF differ in their intrinsic molecular structure so that the former is basically an unstable compound; or (3) human plasma contains some factor(s) which destroys or inactivates AHF. It will be shown that the third possibility is probably correct, since oxalated human plasma can destroy bovine AHF.

METHODS AND MATERIALS

All coagulation procedures were performed in a 37° C. water bath, and uncoated glassware was used exclusively.

AHF activity of specimens was estimated by either of two methods. The first was based on the ability of a specimen to improve prothrombin consumption in the blood of a known hemophiliac and has been described previously.[3] The second was derived from the thromboplastin generation test of Biggs and Douglas.[8] This test has been modified by our laboratory and differs from the original method in the following respects: (1) Only oxalated plasma is used. (2) A stock supply of platelets is prepared from a pint of whole blood. These platelets are kept in deep-freeze in separate small test tubes so that the day's supply can be thawed as needed. (3) Plasma is adsorbed with barium sulfate instead of with aluminum hydroxide. The barium sulfate precipitated from 0.1 ml. each of molar

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Na_2SO_4 and $BaCl_2$ is used per milliliter of plasma, and adsorption is carried out for 30 minutes at room temperature. (4) The substrate used to assay the generated thromboplastin consists of equal volumes of deprothrombinized and Seitz-filtered beef plasma,[9] and a 10 per cent solution of normal plasma in distilled water. (5) Substrate is recalcified 15 seconds before the generating mixture is added. In the thromboplastin generation test AHF activity of a specimen was estimated by substituting it as a reagent in place of the barium sulfate–adsorbed normal plasma in the generating mixture. In the present study no attempt was made to obtain quantitative determination of AHF; only relative activity was determined.

Bovine plasma was obtained from the blood of freshly slaughtered cattle. Nine volumes of blood were added to one volume of 0.1 molar potassium oxalate. Oxalated human plasma was obtained from the blood of normal volunteers, and citrated plasma was prepared from ACD bank blood and from the blood of laboratory personnel. When one-tenth volume of 3.8 per cent sodium citrate was used as an anticoagulant, the results were similar to those obtained from ACD plasma.

EXPERIMENTAL

To demonstrate the difference in storage-lability between oxalated and citrated human plasma, specimens were aged at refrigerator temperature. After the desired period of aging, these specimens were tested for AHF activity by their ability to improve prothrombin consumption in hemophilic blood. In each case, the test plasma was diluted with isotonic saline solution so that when 0.1 ml. of the diluted plasma was added to 1.9 ml. of hemophilic whole blood the final concentration of test plasma was 2 per cent. Fig. 1 shows the results of these studies. Oxalated plasma consistently showed an appreciable loss of AHF activity within 24 hours, and after 7 days it was virtually inactive. A few specimens of citrated plasma appeared to show rapid loss of AHF, but generally the activity was well maintained even after 40 days or more of storage. The loss of AHF activity in the citrated plasmas was not due to low initial AHF content, since these plasmas had been tested when fresh and had then shown normal activity.

Studies on Mixtures of Human and Bovine Plasma.—To account for the difference in storage lability between oxalated specimens of human and bovine plasma, mixtures of these plasmas were studied. After preliminary studies, it was found that 4 volumes of human plasma added to 1 volume of bovine plasma gave the clearest results, and the studies to be reported are based on this proportion.

Mixed human and bovine plasma was allowed to stand at room temperature for the desired period of time. At the same time, control specimens were similarly aged. These consisted of plain human plasma, plain bovine plasma, bovine plasma mixed with four volumes of saline solution, and human plasma mixed with one-quarter volume of saline solution. At the end of the aging period all specimens were tested for AHF activity. In addition, AHF activity was studied in a mixture of human and bovine plasmas which had been allowed to age separately and had been mixed just prior to testing.

Table I and Fig. 2 shows the results of these studies, which confirm the storage stability of bovine plasma. As tested by the ability of specimens to improve hemophilic clotting, the loss of AHF in a mixture of human plasma with bovine plasma is greater than that in bovine plasma mixed with saline solution. Although three weeks of incubation are required to demonstrate this effect clearly, the trend was evident after one week. In the mixture of bovine and human plasmas which had been incubated separately and mixed just prior to

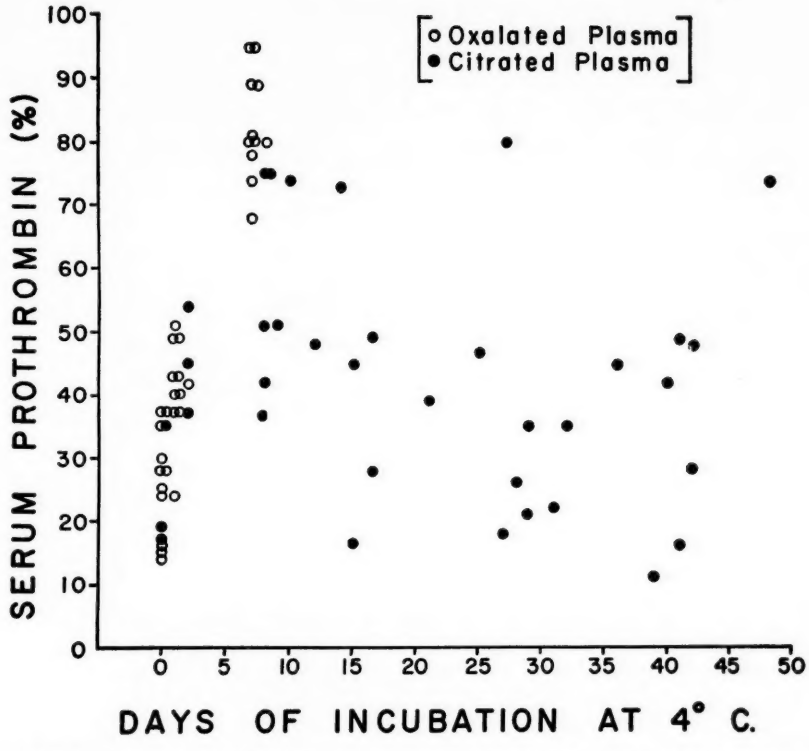

Fig. 1.—Loss of AHF activity in stored human plasma. The AHF activity of a plasma specimen is proportional to its ability to reduce the serum prothrombin of hemophilic blood. A high serum prothrombin indicates low AHF activity.

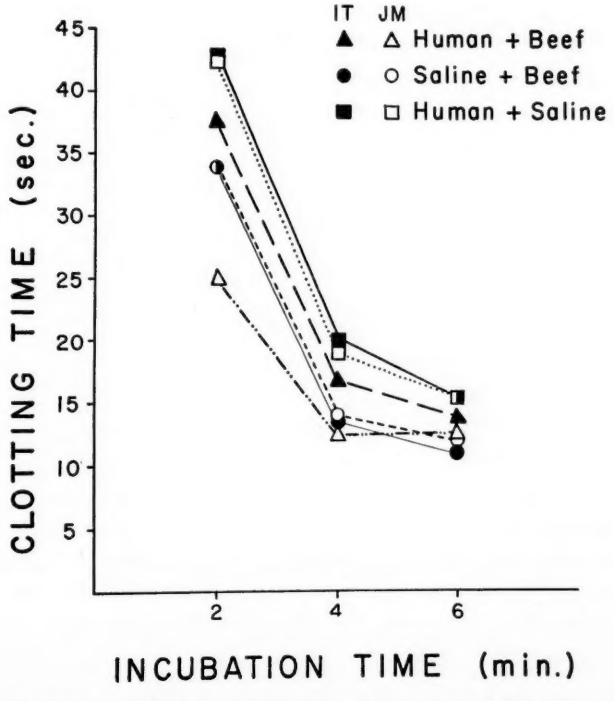

Fig. 2.—Thromboplastin generation curves of aged plasmas and mixtures. *IT*, Incubated together as a mixture; *JM*, incubated separately and mixed just prior to testing.

TABLE I. EFFECT OF HUMAN PLASMA ON BOVINE AHF

SPECIMEN TESTED	PER CENT SERUM PROTHROMBIN OF HEMOPHILIC BLOOD
4 volumes of saline/1 volume of bovine plasma	7
1 volume of saline/4 volumes of human plasma	54
4 volumes of human plasma/1 volume of bovine plasma (incubated together)	42
4 volumes of human plasma/1 volume of bovine plasma (incubated separately, mixed just prior to testing)	9
4 volumes of hemophilic plasma/1 volume of bovine plasma (incubated together)	40
4 volumes of hemophilic plasma/1 volume of bovine plasma (incubated separately, mixed just prior to testing)	10

Specimens were stored for three weeks at room temperature, and tested for AHF activity by their ability to improve prothrombin consumption in hemophilic whole blood. One-tenth milliliter of test mixture was added to 1.9 ml. of hemophilic blood.

testing, the AHF activity was at least as great as that of the bovine plasma incubated with saline solution. Similar results were obtained when the thromboplastin generation test was used as the assay method, but only five days of incubation were required to demonstrate the effect of human on bovine plasma. With both methods the findings were repeatedly confirmed, although the magnitude of difference obtained was variable.

On the basis of these findings the conclusion appears justified that human plasma can destroy the AHF activity of bovine plasma. The loss of AHF in the mixture of human and bovine plasmas cannot be due to the loss of AHF in the human plasma alone, since the AHF activity of the mixture is less than that of bovine plasma mixed with inert saline solution. Likewise, the effect cannot be due to the development in stored human plasma of an anticoagulant. This possibility is excluded by the high degree of AHF activity exhibited by bovine plasma recently mixed with stored human plasma. Actually the AHF activity of such a mixture appeared to be slightly greater than that of bovine plasma incubated with saline solution, probably because of a small residual amount of AHF in the stored human plasma.

Of interest is the finding that hemophilic plasma appears to have the same ability to destroy bovine AHF as does normal human plasma.

Although the nature of the agent(s) in human plasma which inactivates bovine AHF is not established, for the sake of convenience it will be designated as "AHF inactivator" (AHFI) in the remainder of the present study.

Properties of AHF Inactivator (AHFI).—Cohn fractions* of human plasma were tested for AHFI activity. In each case, the fractions were reconstituted to their original plasma concentration, isotonic saline solution having been used as the solvent. Four volumes of the solution were mixed with 1 volume of bovine plasma, and the mixture was allowed to age for five days at room temperature. AHF activity was then measured by the thromboplastin generation method. As seen in Fig. 3, only Fraction III appears to have AHFI activity. However, the AHFI activity of Fraction III may represent a different process from that obtained with whole human plasma, for Fraction III had some inhibitory effect on bovine AHF when tested after immediate mixing.

*Kindly provided by the Cutter Laboratories, Berkeley, Calif.

The following blood derivatives were also tested for AHFI activity:

Serum was obtained from normal blood one hour after clotting had occurred. It was then mixed with one-tenth volume of 0.1 molar potassium oxalate, and again incubated for 15 minutes at 37° C. Such serum had no demonstrable thrombin activity.

Ether extracted plasma was prepared by extracting plasma with diethyl ether in a mechanical shaker. Three separate extractions were performed, each consisting of five volumes of ether allowed to act for thirty minutes.

Heated plasma was prepared by incubation at 60° C. for 15 minutes.

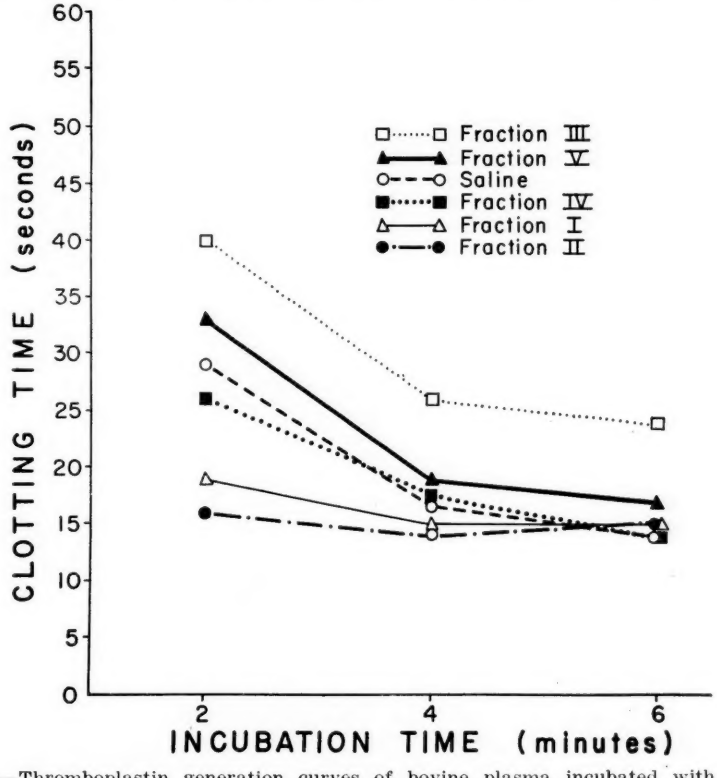

Fig. 3.—Thromboplastin generation curves of bovine plasma incubated with human plasma fractions.

Barium sulfate adsorbed plasma was obtained according to the procedure described in Methods.

Dialysed plasma was prepared by continuous agitation of plasma in a Viskind bag against three changes of saline solution over a period of one hour.

Each of these reagents was mixed with bovine plasma in a 4:1 ratio as described previously, and the resulting mixture was tested for AHF activity after five days of aging at room temperature, by the thromboplastin generation test.

Fig. 4 shows the results of these studies. AHFI was not removed by barium sulfate or ether, but some activity was lost in serum and heated plasma. Most strikingly, dialysis removed all AHFI activity.

Since AHFI appeared to be a small, dialysable molecule, activity was sought in urine. Urine specimens were decalcified with one-tenth volume of 0.1 molar potassium oxalate, and the pH was adjusted to 7.2 with powdered sodium bicarbonate. Four volumes of urine were added to 1 volume of beef plasma, and after five days of incubation at room temperature AHF activity was measured by the thromboplastin generation test. A control study was run with oxalated saline solution used in place of oxalated urine. As seen in Fig. 5, urine has a high degree of AHFI activity, whereas oxalated saline solution has none.

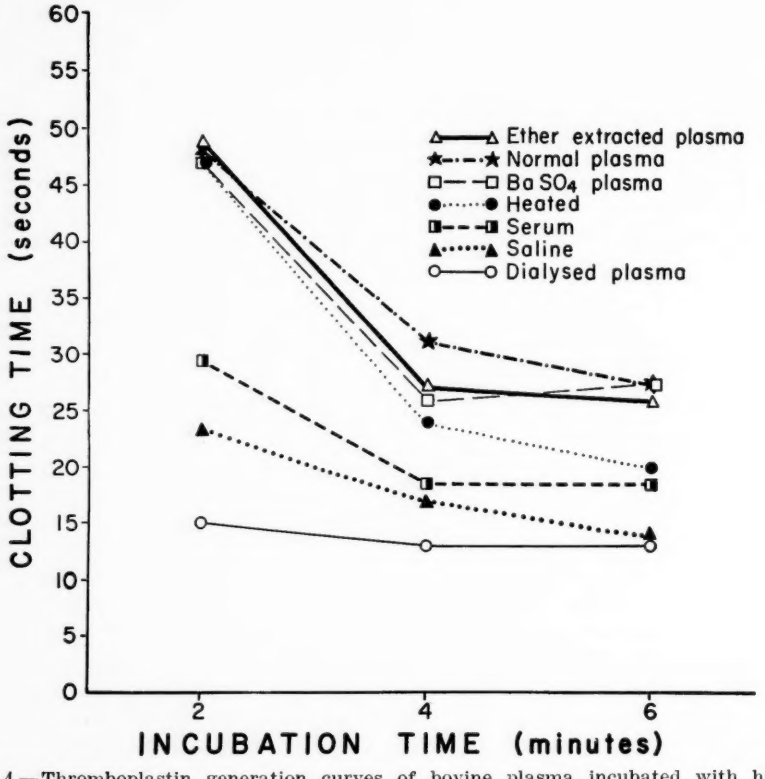

Fig. 4.—Thromboplastin generation curves of bovine plasma incubated with human plasma subjected to various manipulations.

Is AHFI Responsible for AHF Loss in Stored Oxalated Human Plasma?—Since AHFI could be removed by dialysis, the stability of AHF in dialysed human plasma was studied. Rapid dialysis was accomplished as described above, and the following aliquots of the same plasma specimen were studied: (1) Plasma dialysed against isotonic saline solution. (2) Plasma dialysed against isotonic saline solution to which had been added one-tenth volume of 0.1 molar potassium oxalate. (3) Plasma to which had been added one-tenth volume of isotonic saline solution, a dilution approximating that which occurred during dialysis. (4) Untreated plasma. All specimens were then allowed to age at room temperature, and were tested for AHF activity by their action on hemophilic blood.

TABLE II. EFFECT OF DIALYSIS ON AHF STABILITY IN STORED, OXALATED HUMAN PLASMA

SPECIMEN ADDED	PER CENT SERUM PROTHROMBIN OF HEMOPHILIC BLOOD
Saline solution only	100
Fresh plasma	37
Plasma diluted with one-tenth volume of saline solution	100
Plasma dialysed against saline solution	45
Plasma dialysed against oxalated saline solution	40
Plasma dialysed against saline solution after storage	100

Specimens were stored at room temperature for one week and tested for AHF activity by their ability to improve prothrombin consumption in hemophilic whole blood. One-tenth milliliter of each plasma specimen was added to 1.9 ml. of hemophilic blood. The plasmas were diluted such that the final concentration was 2 per cent.

The results of these studies are presented in Table II. Again the marked loss of AHF in stored oxalated plasma is evident and is not influenced by the mere addition of saline solution. However, a marked increase in storage stability resulted from dialysis, whether or not oxalate was removed from the plasma. It was not possible to restore AHF activity to stored plasma by dialysis.

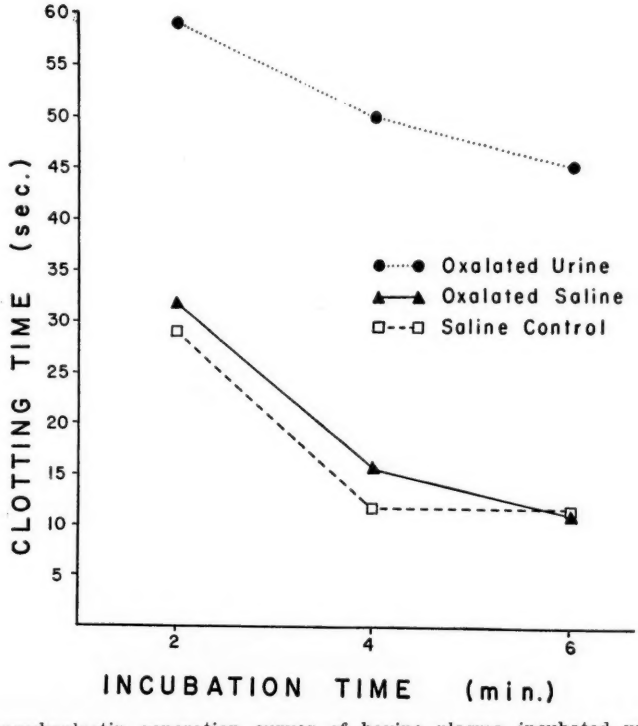

Fig. 5.—Thromboplastin generation curves of bovine plasma incubated with urine or saline solution.

It thus appears that AHFI is responsible for the loss of AHF in stored, oxalated human plasma. Moreover, the loss of AHF during storage cannot be merely the result of direct oxalate action.

Mechanism of AHFI Action.—Preliminary studies were undertaken to determine the means by which AHFI destroyed AHF activity. Various alterations of oxalated plasma were employed in an effort to block AHFI activity.

The following manipulations were tried: (1) Potassium permanganate served as an oxidizing agent. Concentrations between 0.1 and 0.001 per cent were added to plasma. This compound appeared to increase AHF lability. (2) Exclusion of oxygen was attempted by storing plasma under oil with and without separation of the erythrocytes, and also by the addition of different concentrations of reducing agents (sodium bisulfite, ascorbic acid). (3) The pH of plasma was brought to various levels between 5 and 9. Although there appeared to be a slight gain in AHF stability at a pH of 6.3 to 6.9, lability was increased at the extremes of the range. (4) A series of enzyme inhibitors were added to plasma. These included sodium cyanide, potassium ferricyanide, formalin, sodium sulfadiazine, Benadryl, cysteine hydrochloride, detergents, α bromacetic acid, β propiolactone, and sodium dehydroacetate. In no case was a significant degree of increased AHF stability achieved. These studies have provided no clue as to the mechanism of AHFI activity.

DISCUSSION

The increased stability of AHF in citrated plasma has obvious practical application. Since only rare plasmas show marked loss of AHF activity in bank blood, this type of blood will usually be effective in the management of hemorrhagic episodes in hemophiliacs. Fresh blood or plasma is more reliable because of the occasional stored specimen with low AHF activity; but for emergency use, even plasma from outdated blood may be satisfactory.

Of additional interest is the significance of the storage stability of AHF in citrated plasma with respect to purifying this protein. In the past the poor yield of activity in plasma fractions has been attributed to loss from instability. However, it has been shown that even with the greatest precautions to ensure rapid fractionation, little more than 30 per cent AHF activity can be recovered in Fraction I.[10] If Fraction I plus III is tested for AHF activity, the yield is quantitative. Since Fraction III has been shown to have AHF activity,[11] it is evident that at least part of the reason for the poor yield in Fraction I is inappropriate separation rather than deterioration during processing. With citrated plasma, prolonged fractionation procedures may be considered which were hitherto thought to be unfeasible.

Stable as AHF generally is in citrated plasma, a few specimens show rapid deterioration of activity, and many lose some activity. It is probable, although not proved, that antihemophilic factor inactivator (AHFI) may be responsible for this loss of AHF as it is in oxalated plasma. Since AHFI can be removed by dialysis, perhaps all plasma to be used as a source of purified AHF should undergo preliminary dialysis to ensure maximal AHF stability.

Studies designed to purify and identify AHFI are in progress. It is ultimately hoped to find some agent which will counteract its action and thus facilitate stabilization of AHF in human plasma.

SUMMARY

Human antihemophilic factor (AHF) has been found to be considerably more storage-stable in citrated than in oxalated plasma. The storage lability

of AHF in oxalated plasma appears to result from the action of an inactivator (AHFI). This inactivator destroys the normally stable AHF of bovine plasma and is a relatively heat-stable, dialysable substance. Dialysis of oxalated human plasma increases the storage stability of AHF.

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RESISTANCE STRAIN GAUGES APPLIED TO RESPIROMETRY: A NEW METHOD FOR STUDYING RESPIRATION IN THE NEWBORN INFANT

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INTRODUCTION

Respiration in adult human beings can be studied satisfactorily by a variety of techniques, the Tissot-tank type of respirometer and the rubber-bellows pneumograph being perhaps the most commonly used. Respiration in infants, particularly in the newborn age group, cannot be so easily measured, due to the necessity for eliminating discomfort and interference with movement, and to the lack of cooperation on the part of the subjects. Nonetheless, several workers have attempted to study respiration by a variety of methods in newborn infants. A brief review is to be found in the excellent monograph by Smith.[1] He points out particularly the shortcomings of direct visual observations of respiratory rate, since under many conditions a large proportion of the respiratory movements are imperceptible. On the other hand, elaborate apparatus which necessitates application of a mask to the infant's face, and the measurement of expired air in a conventional respirometer, may be sufficiently disturbing to the infant to result in artificially rapid rates being recorded. Early investigators,[2-5] employing a careful balance of these two techniques, found average respiratory rates of about 60 respirations per minute in resting newborn infants. They considered these rates to be too high, due to the above-mentioned effects. A more recent study by Miller and Behrle[6] using a rubber-bellows pneumograph as well as a face mask resulted in average rates which, from their published graphs, are in the neighborhood of 50 to 60 respirations per minute.

In another series of studies,[7-12] a technique was used in which the infant was placed in a body plethysmograph, an airtight chamber from which the head protruded through a rubber collar carefully applied to the neck. After a period of acclimatization, the subjects were reported to be able to sleep or rest quietly without a cumbersome mask. The interior of the plethysmograph was connected to a delicate respirometer, usually of the Krogh type, so

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that every respiratory movement of the infant was recorded by the displacement of the air surrounding its body. By this method average rates of about 45 respirations per minute were recorded.

Enough has been said to stress the difficulties involved in studying neonatal respiration, and to indicate the need for a device which will measure an infant's respiratory movements accurately, and yet not act as a stimulus or restraint. We became acutely aware of this need when we decided to record respiration as part of our current studies of autonomic reactivity in infants. We have been very careful throughout these studies to ensure that whatever measurements we made were of negligible importance as stimuli to the infant. Being unable to find a technique for recording respiration which was acceptable from this point of view, we developed the strain-gauge respirometer described in the following section.

DESCRIPTION OF RESPIROMETER

The advent in recent years of the resistance strain gauge[13] provides investigators in biology and medicine with a valuable tool for measuring small movements with a minimum of gadgetry attached to the subject. Strain gauges are available in a wide variety of sizes, shapes, and materials.[14] They have not yet been as widely adopted in biological laboratories as their potential usefulness would seem to justify.

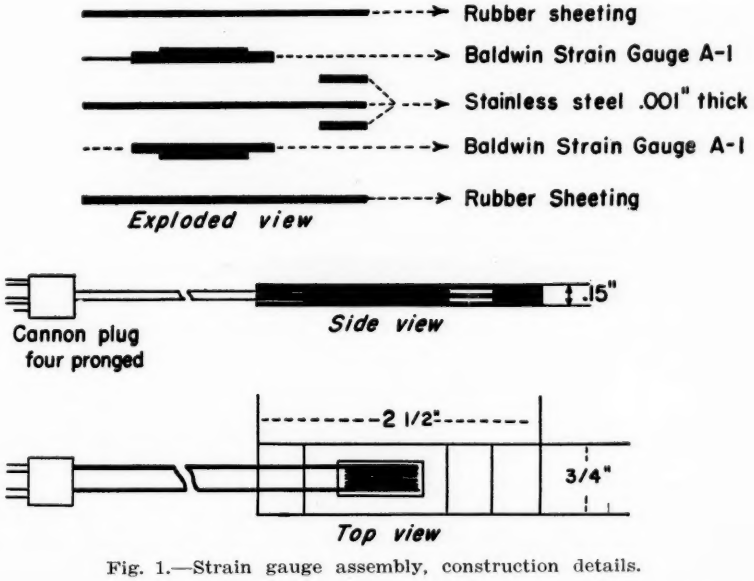


Fig. 1.—Strain gauge assembly, construction details.

In its present form, the respirometer consists of two Baldwin SR-4 type A-1 constantin wire strain gauges, cemented to opposite sides of a one-mil (0.001 inch thick) strip of stainless steel, as shown in Fig. 1. The different regions of the metal strip are given different stiffnesses by winding with plastic tape, so that the flexural movements are localized at the region where the sensitive portions of the gauges are. The whole assembly is wrapped in latex sheeting to make it comfortable to the skin, and taped to the subject with plastic or adhesive tape. Electrical connection to the gauge assembly is made by a light, braided cable, terminating in a Jones plug, as shown in Fig. 2. It has been found very easy to keep this cable out of the infant's way, and also outside

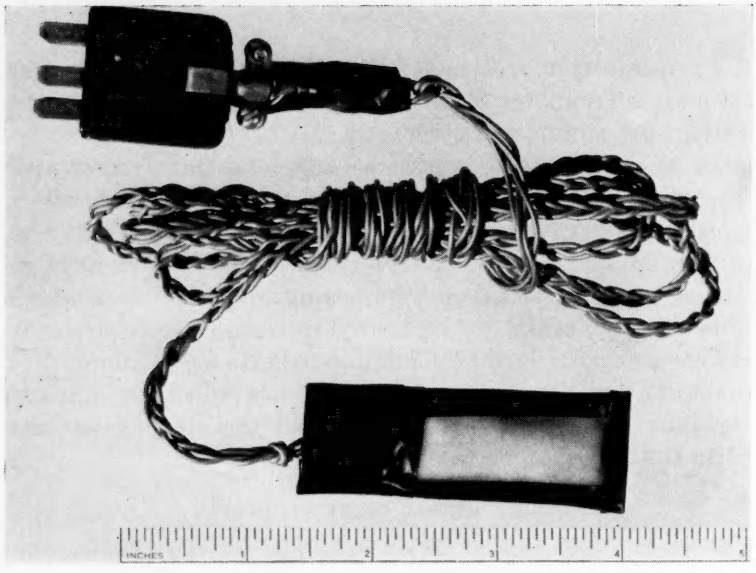
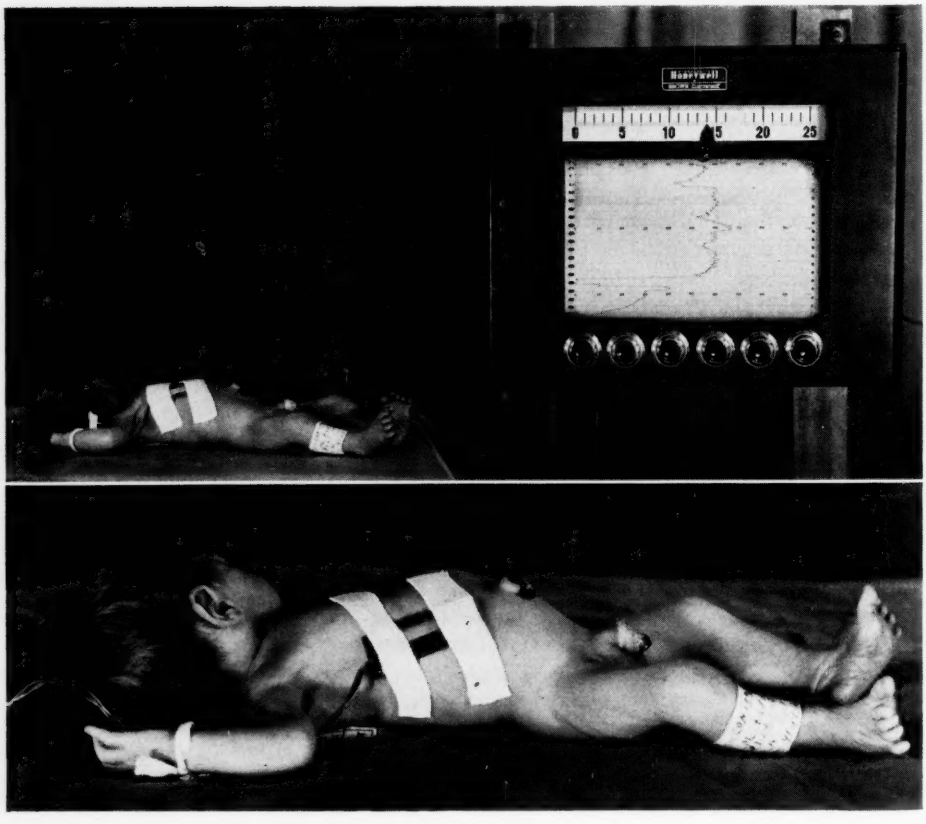


Fig. 2.—Photograph of strain gauge assembly.

A.



B.

Fig. 3.—*A* and *B*, Strain gauge assembly taped to infant.

his sphere of attention, so that there is a mutual lack of interference between gauge and subject. The best location seems to be across and at right angles to the costal margin at the side, as shown in Fig. 3, *A* and *B*.

The two gauges are connected differentially into a standard D.C. bridge circuit, as indicated in Fig. 4. The output of the bridge is fed into a type 153 × 18 adjustable sensitivity Brown recorder. Small flexural movements of the assembly produce dimensional changes, and therefore resistance changes, in the wires of the strain gauges, and result in a voltage output from the bridge circuit which is a function of the degree of bending of the whole assembly. Since this output voltage is small, on the order of a few millivolts, a sensitive recorder is used. This expensive item can be eliminated, however, by using an A.C. source for the bridge, amplifying the output in a few stages of conventional amplification, and then demodulating. By means of appropriate circuitry, the resulting signal can be made adequate to drive any available recorder. The circuits required are not complicated, and can easily be built by a competent electronics technician.

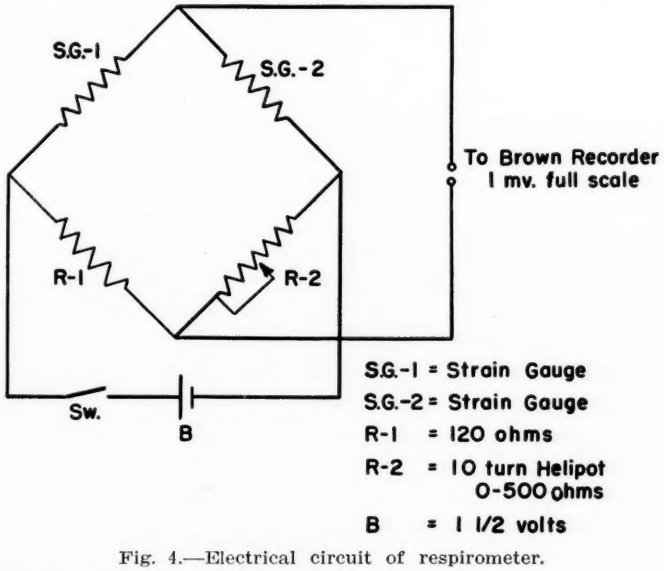


Fig. 4.—Electrical circuit of respirometer.

One disadvantage of the strain gauge arises from the fact that it was not designed to be flexed through such large angles as are necessarily encountered in this application, particularly in applying and removing the gauge assembly. As a result, the life of any one assembly is limited to between thirty and forty runs of about three hours each. Since the gauges are relatively inexpensive, and the assembly is simple to make, this has not proved so far to be a serious drawback.

RESULTS AND DISCUSSION

To indicate the wide range of usefulness of this respirometer, Figs. 5 through 8 show a series of typical records taken from a variety of subjects.

Figs. 5 and 6 were taken on two adults, under the conditions indicated in the figures. The difference in breathing pattern between these two normal adults is striking, and was correlated with clinical observation.

Fig. 7 shows some typical respiration records from a 5-day-old infant under various conditions. The regularity and slow rate of the sleeping pattern form an interesting contrast to the feeding pattern with its irregularity

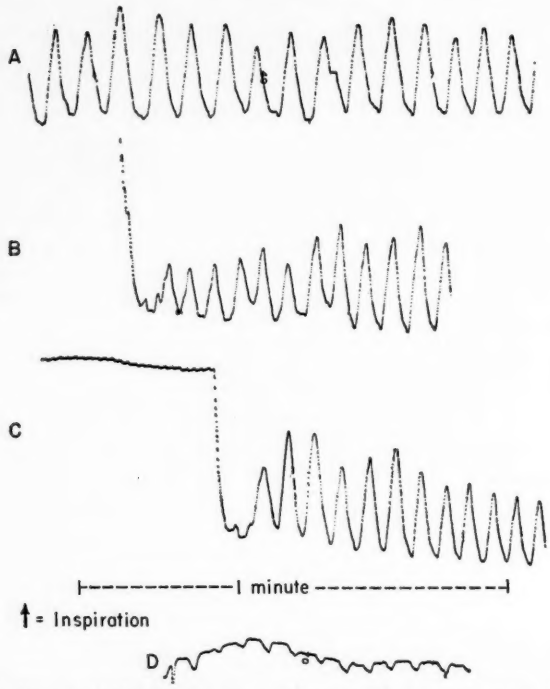


Fig. 5.—Respiration records taken on a 24-year-old normal man (N. H. G.). Gauge assembly No. 4. *A*, Lying on back, wide awake; *B*, moderate hyperventilation (not shown) followed by gradually increasing amplitude of breathing, on back; *C*, holding breath, followed by gradually decreasing amplitude of breathing, on back; *D*, sitting erect.

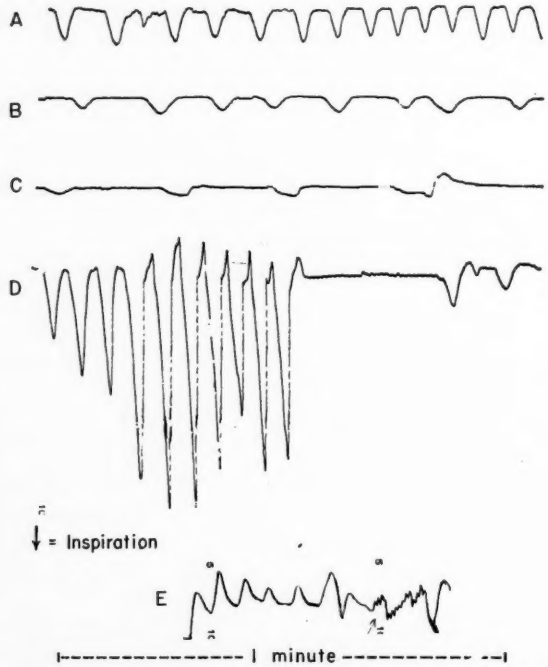


Fig. 6.—Respiration records taken on a 33-year-old normal man (P. S.). Gauge assembly No. 5. *A-D*, Lying on back; *A*, wide awake; *B*, awake but drowsy; *C*, asleep; *D*, hyperventilation followed by apnea. *E*, Sitting erect.

in frequency. The pattern of Fig. 7, *C* illustrates the fact that the respirometer also records bodily movements in addition to respiration. At first this was regarded as a disadvantage, but since the patterns are fairly easily resolved, it has actually turned out to be an advantage in some circumstances, particularly in studies of infant activity.

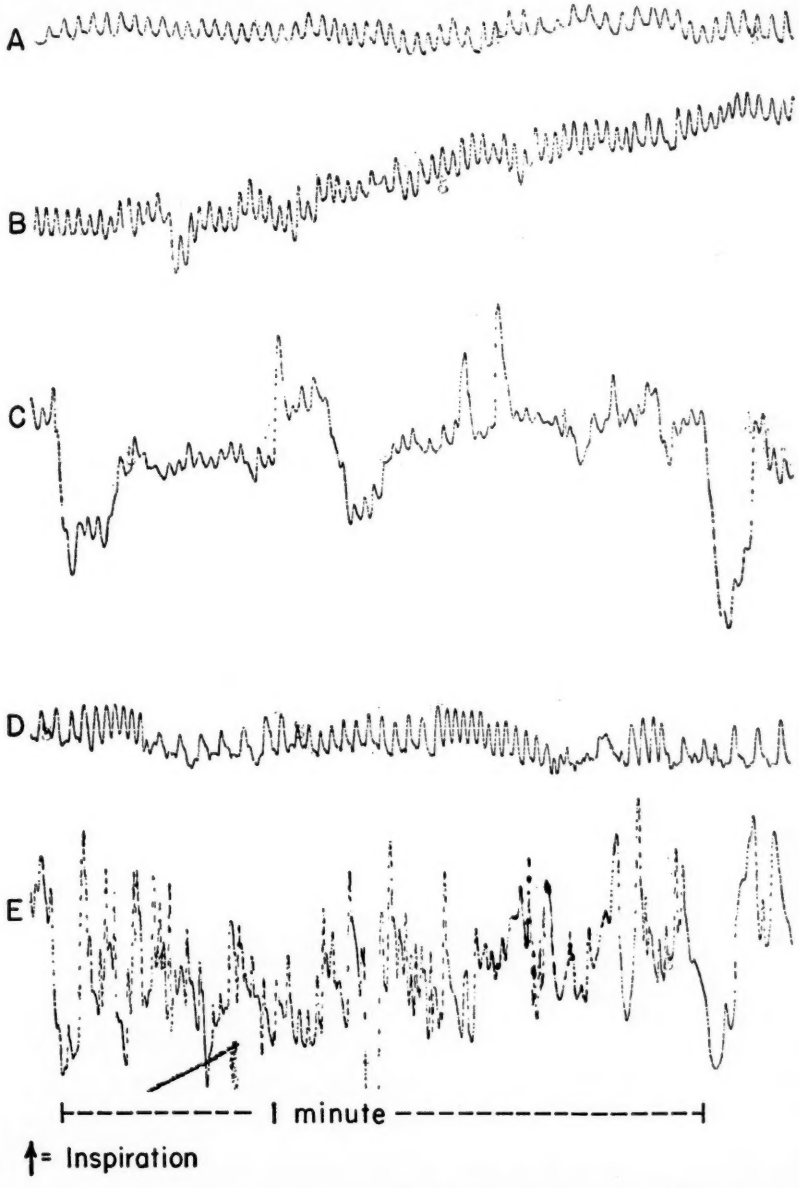


Fig. 7.—Respiration records taken on a 5-day-old infant (No. 29) lying on back. *A*, Asleep; *B*, awake, but very quiet; *C*, awake and moving about; *D*, feeding from bottle. Periods of rapid breathing (80 per minute) are interspersed with periods of sucking, swallowing, and partial apnea. *E*, Crying lustily; rapid respiration interspersed with cries and body movements.

Fig. 8 is part of a record taken on an anencephalic infant. It shows clearly an abnormal lack of synchrony between diaphragmatic and thoracic breathing.

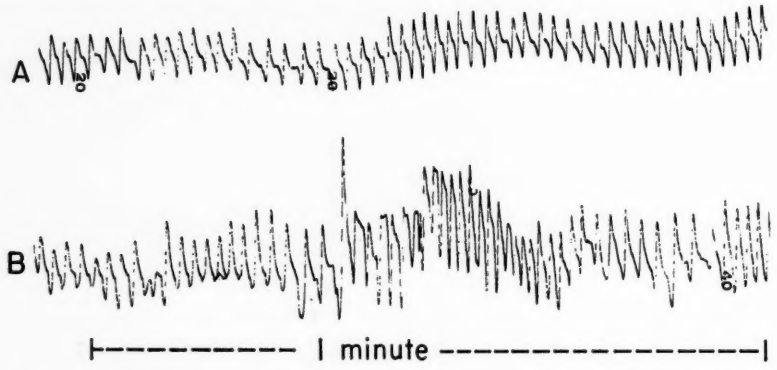


Fig. 8.—*A* and *B*, Records taken from an anencephalic infant 2 weeks old. The main peaks are due to diaphragmatic breathing, the variable humps on the expiratory side are due to nonsynchronized thoracic breathing.

Although it is clear from such records as shown in Fig. 7 that an infant's average respiratory rate may have a somewhat limited significance, it is of interest to compare the rates obtained in these studies with previously published results. The average rate for the infant whose records are shown in Fig. 7 was 50 respirations per minute when asleep, 65 when awake and quiet, and 46 during feeding from a bottle. In a series of nine 3- or 4-day-old infants, all measured average respiratory rates fell within the range from 30 to 65 respirations per minute. The rates obtained with this technique, therefore, agree well with those results cited above, which ranged from 45 to 60 or more.

SUMMARY

To meet the problem of measuring respiration in very young infants, a respirometer has been devised which uses resistance strain gauges as its sensitive elements. Due to its light weight and small size, this device causes negligible discomfort or restraint. The respirometer is described, and records indicating its applicability are presented.

We wish to acknowledge the continuing encouragement of Dr. R. W. Gerard, Director of the Neurophysiological Laboratories, without whose help and support this work could not have been accomplished.

We are also grateful to the Director of the Aeromedical and Physical Environment Laboratory, Dr. J. P. Marbarger, for his cooperation, and for providing space and facilities for much of the experimental work.

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ISOLATION OF SALMONELLA TYPHI FROM THE BLOOD STREAM

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ISOLATION of *Salmonella typhi* from the blood stream is by far the most satisfactory method of diagnosis in the early stages of typhoid fever since a bacteremia is present during the first few days of illness. Isolation of the causative organism from stools and urine is of no help at this time, and serologic procedures rarely give much definite information. Indeed, as regards the latter, the widespread use of T.A.B. vaccine has rendered the interpretation of agglutinin titers a matter of some difficulty. In particular the level of flagellar agglutinins, in those previously vaccinated, is of very doubtful significance in any illness characterized by pyrexia.[1, 2]

Isolation of *S. typhi* from the blood may be accomplished in three ways, viz.: (a) by culture of whole blood in some suitable medium, usually a bile-containing one, (b) by culture of blood clot after removal of the expressed serum, and (c) by culture of bone marrow. We have no experience of the third method, but it is claimed to give satisfactory results.[3, 4] We have, however, made a study of some of the factors governing successful isolation of *S. typhi* from whole blood and blood clot, in an area where typhoid fever is endemic.

METHODS AND RESULTS

1. *Procedure.*—Blood cultures were performed by adding 8 to 10 ml. of whole blood, obtained by venipuncture, to 35 ml. of 0.5 per cent taurocholate broth in 4-ounce screw-capped sterile bottles. The culture bottles were incubated at 37.0° C. and subcultures were plated daily onto Wilson and Blair's medium. All culture bottles were kept for 3 weeks before being discarded. Later in the investigation the volume of culture medium was increased to 50 ml., when it became evident that 35 ml. was insufficient.

In the case of clot cultures the separated serum was removed from 5 ml. volumes of clotted blood collected in sterile 3 inch by ½ inch tubes. The residual clot was then added to 15 ml. of streptokinase bile salt broth (containing 100 units per milliliter of streptokinase) in universal containers. Streptokinase produces rapid clot lysis with release of organisms trapped in the clot, as we have previously described.[5] Subcultures were plated daily as in the case of the blood cultures. In each case suspicious colonies were picked off into peptone water and checked for biochemical and serologic reactions.

2. *Results.*—During the period May, 1952, to November, 1953, we used 35 ml. of medium for the blood cultures. Table I shows the findings in the case of 104 patients where simultaneous culture of whole blood and blood clot was carried out.

The superiority of the clot culture technique is only too evident and suggested to us that the presence of serum in sufficient concentration may adversely

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affect the percentage of positive isolations. More detailed investigation has proved this to be so, and we have shown that where blood clot is cultured without removal of the serum, in streptokinase broth, the results are inferior to culture of the clot with the serum discarded.[6]

TABLE I. CULTURE OF WHOLE BLOOD AND BLOOD CLOT

NO. OF CASES WHERE BOTH WHOLE BLOOD AND BLOOD CLOT CULTURED. (ONE OR BOTH POSITIVE)	BLOOD CULTURE		CLOT CULTURE	
	+	−	+	−
104	52	52	98	6

Between November, 1953, and December, 1954, we increased the volume of whole blood culture medium to 50 ml. and the results in Table II were obtained, again using the clot culture results for comparison since the method of performing the latter was constant throughout.

TABLE II. CULTURE OF WHOLE BLOOD AND BLOOD CLOT

NO. OF CASES WHERE BOTH WHOLE BLOOD AND BLOOD CLOT CULTURED. (ONE OR BOTH POSITIVE)	BLOOD CULTURE		CLOT CULTURE	
	+	−	+	−
71	57	14	68	3

It will be seen that of the 71 cases, there were 57 positive whole blood cultures, i.e., 80 per cent, as compared with 95.7 per cent in the case of the clot cultures. This compares with 50 per cent for whole blood cultures in Table I, where the volume of medium was less. As regards the 3 negative clot cultures in Table II, the volume of blood submitted for examination in these cases was only 2 to 3 ml. as against 8 to 10 added to the whole blood culture medium.

EFFECT OF MEDIUM VOLUME

Mackie and Finkelstein[7, 8] showed that the bactericidal effect of normal serum was dependent on the combined action of naturally occurring antibody and complement and that gram-negative organisms were more susceptible to killing by this means than gram-positive ones. Gerstung,[9] in a series of 60 combinations of sera with various organisms, found that only 29 yielded growth at 1/12 or greater concentration of serum. We have shown that with a blood/medium ratio of more than ¼–⅕ the chances of achieving a positive isolation are correspondingly diminished.[6] Comparison of the findings in Tables I and II shows clearly the benefit to be derived from providing a volume of medium sufficient to dilute out any bactericidal serum factors which may be present. It is evident that even 50 ml. is not adequate since the results are inferior to the clot culture technique. Most workers have advocated large volumes of culture medium. Pulvertaft[10] added 10 ml. of blood to at least 5 times that volume of blood, and Murray and Kalz[11] recommended 190 ml. of medium. That the role of bactericidal serum factors is not the only one, however, which may influence successful isolation is shown by the fact that positive results have

been claimed with volumes of medium where the dilution factor is small. Batty, Shaw and Mackay,[12] for example, obtained 71 positive cultures in 76 cases by adding 5.0 ml. of blood to only 15 ml. of bile broth.

EFFECT OF STRAIN OF ORGANISM

Not all strains of *S. typhi* will exhibit the same degree of sensitivity to the bactericidal action of serum, and the following investigation was undertaken to demonstrate the different response of different strains of *S. typhi* to the same serum.

1. *Procedure.*—Twenty milliliters of blood from a donor with no prior history of T.A.B. vaccination was collected in a sterile Erlenmeyer flask containing glass beads and defibrinated by shaking. After centrifugation, the serum was removed and 1 ml. used to determine agglutinin levels against standard suspensions of the 0.901 and H.901 strains. No agglutinins were found in this particular serum.

TABLE III. EFFECT OF SAME SERUM ON DIFFERENT STRAINS OF S. TYPHI

STRAIN	INITIAL INOCULUM	TIME OF INTERACTION		
		10 MINUTES	2 HOURS	24 HOURS
	4,200	50	14	C
	420	10	4	P
5	42	2	nil	nil
	8,400	180	82	nil
	840	24	14	nil
94	84	nil	nil	nil
	7,500	200	60	30
	750	60	16	4
150	75	10	nil	nil
	2,000	28	nil	nil
	200	2	nil	nil
151	20	nil	nil	nil
	4,200	150	64	16
	420	60	22	2
160	42	2	nil	nil
	5,100	10	nil	nil
	510	2	nil	nil
2329	51	1	nil	nil
	6,200	100	80	C
	620	48	30	C
48	62	2	2	P
	1,800	150	82	C
	180	10	4	C
41	18	2	1	P
	2,200	50	10	nil
	220	21	3	nil
57	22	nil	nil	nil
	1,600	40	12	nil
	160	3	6	nil
620	16	1	nil	nil

Figures in time columns = No. of colonies per 0.02 ml. volume.
C = confluent growth.
P = single colonies too numerous to count.

One-half milliliter volumes of the remaining serum were placed in sterile 3 inch by ½ inch tubes along with 0.4 ml. of 1.0 per cent bile broth. One-tenth milliliter volumes of each of 3 suitable tenfold dilutions of an 18-hour broth culture of a freshly isolated strain of *S. typhi* were added to each of the first 3 tubes, 1 dilution to each tube. The fourth tube served as an uninoculated control. The dilution of the serum in the first three tubes was thus ½. The bacterial content of each dilution was checked by plate count. Tubes were incubated at 37.0° C. and 0.02 ml. volumes were seeded onto the surface of dried nutrient agar plates after 10 minutes, 2 hours, and 24 hours, according to the technique of Miles and Misra.[13] Six spots were seeded in each case and the average colony count was calculated after overnight incubation of the plates at 37.0° C.

2. *Results.*—Table III shows that only 2 of the 10 strains tested produced growth of viable organisms, after 24 hours, with each of the bacterial dilutions employed. Strain 5 produced growth with the two highest inocula but not with the lowest, and two other strains, 150 and 160, produced a few colonies after 24 hours' interaction, in the case of the highest inocula. With the remaining strains there was a bactericidal effect evident after 24 hours' interaction, and in the case of 2 strains this effect was noted after 2 hours. The addition of a further 5.0 ml. of broth to those tubes showing no evidence of growth after 24 hours' interaction did not result in any further growth, following incubation at 37° C., suggesting that there had been a true bactericidal effect and not merely a bacteriostatic one. Khairat,[14] in an investigation of the action of serum on a number of organisms, found 1 of 3 strains of *S. typhi* to be susceptible to killing in this manner. Mackie and Finkelstein[8] have shown that "R" variants are more susceptible than the "S" forms.

EFFECT OF VOLUME OF BLOOD

In those diseases characterized by a bacteremia the average number of organisms per milliliter of blood is small.[10] Unless a sufficiently large amount is sampled, the chances of achieving a positive isolation are lessened. The degree of bacteremia in 15 cases of typhoid fever was investigated as follows.

1. *Procedure.*—Ten milliliters of blood from each patient was added to 1 ml. of 3.8 per cent sterile sodium citrate. One milliliter volumes were then placed in sterile Petri plates and 20 ml. of melted and cooled nutrient agar was added. After setting of the agar the plates were incubated at 37.0° C. for 36 hours and the number of colonies calculated per milliliter of blood.

TABLE IV. BACTEREMIA IN CASES OF TYPHOID FEVER

CASE	NO. OF ORGANISMS PER ML.	AVERAGE NO. OF ORGANISMS PER ML.
1	2.5	
2	8.0	
3	4.5	
4	12.0	
5	1.0	
6	0.5	
7	4.0	7.6
8	14.0	
9	1.5	
10	6.0	
11	22.0	
12	13.0	
13	1.0	
14	8.0	
15	6.5	

2. *Results.*—In Table IV the dilution effect of the citrate has been ignored. It will be seen that the average number of colonies per milliliter was small in each instance, and it will be evident that a small blood sample might well be inadequate to produce a positive result, particularly if only a small volume of medium is employed.

DISCUSSION

These considerations indicate the role played by the medium volume, the degree of bacteremia, and the particular strain of *S. typhi* in successful isolation of this organism from the peripheral blood. A number of other factors, however, are concerned and some of these have been discussed by Batty Shaw and Mackay.[12] They include the following: (a) *Stage of Illness.* These workers found that the highest percentage of positive isolation from whole blood culture was obtained in the second and third weeks of illness, namely, 90 per cent and 84 per cent, as compared with 80 per cent in the first week. These findings agree with those of Chatterjee,[15] who recorded 55 per cent, 70 per cent, and 75 per cent for the first three weeks. Stuart and Pullen,[16] however, found the highest number of positive isolations to occur during the first week and quoted figures of 81 per cent, 62 per cent, and 32 per cent for the first three weeks. Our experience is similar to that of Batty Shaw and Mackay,[12] the highest percentage of positive results being found in the second week of illness (Table V).

TABLE V. ISOLATION OF S. TYPHI DURING COURSE OF ILLNESS

TIME	NO. OF CASES	NO. POSITIVE	PER CENT POSITIVE
1st week	25	18	72
2nd week	55	50	91
3rd week	41	35	83
4th week	11	6	54

(b) *Duration of Incubation of Cultures.* Topley and Wilson[17] stated that blood cultures "should not be discarded as negative for at least five days." Butler,[18] on the other hand, recommended culture for 1 month in view of the occurrence of delayed positive results. Batty Shaw and Mackay[12] recorded an average duration of 4 to 5 days' incubation as being necessary but stated that 17 per cent of 71 positive cultures only became so on the ninth to eleventh days of incubation. In our experience of 90 positive whole blood cultures where the volume of medium used was 35 ml., the average duration was 4.8 days with a variation of 1 to 16 days. Of these 90, 10 per cent gave a positive result after more than 10 days' incubation. In the case of 59 positive cultures, however, where the medium volume was 50 ml., the average duration of incubation was only 2.0 days and the variation 1 to 5 days. These figures compare with an average incubation period of 1.9 days in the case of 170 positive isolations from blood clot, using streptokinase bile broth as culture medium.

(c) *T.A.B. Vaccination.* Conflicting reports are to be found regarding the effect of previous T.A.B. vaccination on the isolation of *S. typhi* from blood. We have little experience of this matter since in only 12 of our cases was it possible to obtain a history of such vaccination. In all 12, however, positive results

were readily obtained. Batty Shaw and Mackay[12] are of the opinion that previous T.A.B. has little if any effect on successful isolation and quoted Dick,[19] who found positive cultures in 189 of 238 cases (79 per cent), in previously vaccinated service personnel.

(d) *Degree of Pyrexia.* It is not commonly realized that *S. typhi* may be recovered from the blood stream in spite of the patient being apyrexial. Such isolation has been recorded frequently, however.[12, 20, 21] This is our own experience also.[22]

(e) *Effect of Antibiotic Therapy.* In most of the recorded series to be found in the literature it is stated that the effect of chloramphenicol in typhoid fever has been to sterilize the blood stream in a matter of 24 hours and often much less. In our experience this is not the case. Using the clot culture method described we have been able to recover the organism on 20 occasions in spite of previous administration of chloramphenicol in what are usually considered adequate doses. In one instance isolation was made after a total dose of 22 Gm. on the eleventh day of treatment. In another, isolation was made on the eighth day after a dose of 10 Gm.[23] We believe the mechanism of survival of the organisms in these cases to be intracellular parasitism with protection of the organism from the circulating antibiotic.

SUMMARY

A number of factors concerned in the isolation of *S. typhi* from the peripheral blood are considered. It is shown that the volume of medium must be sufficient to dilute out any bactericidal serum factors which may be present in the culture of whole blood. The nature of the infecting strain will to a large extent determine whether such a bactericidal effect will take place.

The low degree of bacteremia in the average case of typhoid fever makes it necessary to sample as large a volume of blood as possible. The stage of illness and the duration of culture will both play a part in determining the percentage of positive recoveries.

It appears unlikely that previous T.A.B. vaccination has much effect in influencing successful isolation.

Positive isolation may be obtained in spite of the patient being aferbrile and often in spite of the administration of chloramphenicol.

I am indebted to Dr. J. C. Thomas, Provincial Pathologist, Natal Provincial Administration, for his continued interest in this investigation.

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LABORATORY METHODS

PROPOSAL FOR THE DISTRIBUTION OF A CERTIFIED STANDARD FOR USE IN HEMOGLOBINOMETRY

PREPARED BY THE DIVISION OF MEDICAL SCIENCES, NATIONAL ACADEMY OF SCIENCES–NATIONAL RESEARCH COUNCIL

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THERE is probably no procedure more commonly used in clinical laboratories than hemoglobinometry, and few that are less satisfactory in their results. The deficiencies may reside, in part, in inadequacies of the chosen method. In larger part, however, they result from manipulative errors in the measurement and processing of the samples of blood. The latter involve questions of technical proficiency which are entirely within the control of the analyst himself. When, however, he converts his observations—gasometric or photometric readings, measurements of specific gravity, etc.—to grams of hemoglobin in 100 ml. of blood, he must depend upon his own calibration or a calibration supplied by an instrument maker, both procedures involving a conversion factor taken from the literature. To be satisfied that the calibration remains valid for the instrument, reagents, and technical routines in use in his laboratory, he should periodically undertake a precise and time-consuming series of standardizations. If a uniform hemoglobin standard were available nationally and a single method of analysis widely practiced, the individual laboratory would then have assurance not only that its results would be comparable from month to month, but that they would also be comparable with those from other laboratories employing the same standard. Inconsistencies in results that persisted under these conditions could then be clearly attributed to technical and manipulative errors.

The purpose of this report is to describe a plan for the distribution of a certified hemoglobin standard and to advocate the wide adoption of a recommended analytical procedure. The use of both the new procedure and the standard is recommended. At the same time it is pointed out that the standard may be employed for the calibration of other methods of hemoglobin analysis in laboratories that do not choose to adopt routinely the advocated photometric procedure.

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In 1941 the British Medical Research Council instituted an extensive study of the problem of standardizing hemoglobinometry and emerged with (a) a recommended analytical procedure, (b) a simple hemoglobinometer for general use, and (c) a certified national hemoglobin standard for distribution to cooperating laboratories.[1-4]

In the United States the initiative was taken by the Army Medical Service Graduate School with a limited field trial[5] of a cyanmethemoglobin solution proposed by Dr. David L. Drabkin. The success of the Army plan so impressed the Hematology Study Section of the National Institutes of Health that it requested the National Research Council to explore the possibility of establishing a National Hemoglobin Standard for general use throughout the country.

To this end the Academy–Research Council established an *ad hoc* Panel under the Subcommittee on Blood and Related Problems of the Division of Medical Sciences. This Panel has sought the cooperation of the College of American Pathologists, the American Society of Clinical Pathologists, the American Association of Blood Banks, the Department of Defense, the Veterans Administration, the National Institutes of Health, and the National Bureau of Standards. It has also maintained close liaison with the Committee on Hemoglobinometry of the Medical Research Council of the United Kingdom and with the National Research Council of Canada.

The Panel gave serious consideration to the British plan, but decided that in respect to simplicity and adaptability, the cyanmethemoglobin method[6-11] adopted by the U. S. Army would be more suitable for use in the United States and Canada. It was agreed that the choice of a solution of some form of hemoglobin as a standard for hemoglobinometry was both logical and direct. In contrast to a glass standard, it would have the advantage of adaptability to a variety of photometric instruments and cuvettes. Among the forms of hemoglobin well adapted to photometry, cyanmethemoglobin has outstanding advantages. It has been shown that solutions of this pigment are stable for years when preserved at refrigerator temperatures.[12] The absorption band of cyanmethemoglobin in the region of 540 mμ is broad rather than sharp, so that its solutions are suitable for use in filter type photometers as well as in narrow band spectrophotometers.[7, 13] Finally, all forms of hemoglobin likely to be found in blood, with the exception of sulfhemoglobin, are quantitatively converted to cyanmethemoglobin upon the addition of a single reagent.[7]

RECOMMENDATIONS

On the basis of these considerations, the Panel reached agreement on the following recommendations:

1. There shall be a Standard of Reference in the form of a preparation of crystalline human hemoglobin prepared by the method of Drabkin.[14-16] The acceptable criteria for this preparation shall be that a

solution containing 1 milliatom of hemoglobin iron per liter shall have a millimolar extinction coefficient of 11.5 at a wave length of 540 mμ, when measured as cyanmethemoglobin.[7, 13] Certification of this Standard of Reference shall rest upon the results of spectrophotometric measurements and of analysis for iron made independently by the National Institutes of Health, the Army Medical Service Graduate School, the National Bureau of Standards, and Dr. Drabkin's laboratory at the University of Pennsylvania.

Professor King of the Postgraduate Medical School in London will also characterize the Standard of Reference both chemically and spectrophotometrically and compare the results of its use with determinations of hemoglobin employing the British Standard.

2. The iron content of hemoglobin shall be accepted to be 0.335 per cent.[17] This value for iron is the traditional figure used in this country and is in substantial agreement with that adopted by the British. It corresponds with an equivalent weight for hemoglobin of 16,700 per atom of iron and with an oxygen capacity of 1.34 ml. per gram of hemoglobin.[17]

   The adoption of an agreed figure for the iron content is necessary in order that the spectrophotometric measurements in terms of iron may be translated into grams of hemoglobin. Should any change be made in the future in the accepted values for the extinction coefficient of cyanmethemoglobin and the iron content of hemoglobin, results based on the use of the above standard may be readily recalculated.

3. There shall be a Standard for Distribution in the form of a certified solution of cyanmethemoglobin which shall be prepared directly from the Standard of Reference. The Standard for Distribution shall be packaged as three separate solutions containing certified concentrations of approximately 20, 40, and 60 mg. of hemoglobin in the form of cyanmethemoglobin per 100 ml.

   These three solutions will correspond to 1 to 250 dilutions of blood containing approximately 5, 10, and 15 Gm., respectively, of hemoglobin per 100 ml. After bottling, samples of these standard solutions will be spot-checked for correctness of optical density by the four analytical laboratories that have been designated above. The batch will then be certified and distributed to cooperating laboratories by designated national agencies.

4. In conjunction with the use of the proposed standards, it is recommended that clinical laboratories consider the adoption of the cyanmethemoglobin method of hemoglobin determination described by Drabkin.

The use of the cyanmethemoglobin method follows from the concept that it would be logical to adopt a method of analysis which converts hemoglobin into the same pigment as that used in the standard. It does not, however, preclude the use of the cyanmethemoglobin standard for the calibration of other methods of hemoglobin analysis which may be in routine use in some laboratories. However, it should be realized that such a procedure may lead to some loss of accuracy.

The cyanmethemoglobin method employs a single solution containing potassium ferricyanide and potassium cyanide, which converts the hemoglobin in blood quantitatively to cyanmethemoglobin. The ferricyanide converts the hemoglobin iron from the ferrous to the ferric state to form methemoglobin, which then combines with potassium cyanide to produce the stable pigment cyanmethemoglobin. These two reactions are rapid and stoichiometric.

There should be no reluctance to employ this standard and reagent because they contain cyanide. The concentration of cyanide in the reagent that is proposed for use is only 52 mg. of potassium cyanide per liter. Its lethal dose for man approaches four liters.[18] Most clinical laboratories use for the determination of uric acid, a reagent containing 50 Gm. of this salt per liter. In view of this and of the fact that laboratories of clinical pathology are disciplined in the use of such dangerous materials as isotopes and virulent pathogens, it would seem that the handling of the proposed reagent constitutes a quite negligible hazard.

The Panel also agreed to undertake a field trial of one year's duration using the Standard for Distribution described above. It is now felt that the plans for this field trial have progressed to the point where the participation of laboratories desiring to co-operate may be invited. The standards will be prepared by Dr. David L. Drabkin, and distributed without charge to clinical laboratories on application, provided they will agree to meet certain minimum requirements for participation, as follows:

1. To conduct and report at three-month intervals, for one year, measurements of the actual photometric readings of the three standard solutions in the photometer routinely in use for hemoglobin measurements in that laboratory.

2. To cooperate in answering a simple questionnaire designed to furnish information on the influnece of various factors on the results of the hemoglobin determinations which will assist the Panel in its long-range plans for making this standard available on a national scale.

3. To cooperate in the analysis and reporting of (a) an unknown solution of cyanmethemoglobin, and (b) an unknown sample of blood.

The Standard for Distribution, consisting of the three solutions described above will be packaged as a single unit. Details of the procedure for the determination of hemoglobin as cyanmethemoglobin, as well as details of the procedure for calibrating another method in terms of the cyanmethemoglobin standard, will be furnished with the standard.

Distribution will be made to civilian laboratories by the College of American Pathologists, 203 North Wabash Avenue, Chicago, Illinois; to military and government laboratories by the Army Medical Service Graduate School, the Navy Bureau of Medicine and Surgery, the Air Force Surgeon General's Office, and the Veterans Administration Central Office, Room 871; and to laboratories in Canada through the Division on Medical Research, National Research Council, Ottawa, Ontario, Canada. Cooperating laboratories are requested to apply to the distributing agency with which they are most closely associated. Because of limitation in the number of sets of the standard available, distribution will be determined by priority of application and willingness to comply with the conditions listed above. Application for standards will assume acceptance of these conditions.

It is estimated that the Standard will be ready for distribution by April 15, 1955.

This plan has been drafted by the *ad hoc* Panel on the Establishment of a Hemoglobin Standard of the Division of Medical Sciences, National Academy of Sciences–National Research Council.

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ELECTROMETRIC URINOMETRY

A Note on Comparative Determinations of Urinary Osmolarity and Specific Gravity

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THE most widely used simple means of estimating renal function is the determination of specific gravity. This is best applied in concentration tests.[1-4] In these, restriction of water intake with maintenance of urinary solute load results in formation of urine of maximum specific gravity, which is used as the measure of concentrating power.

This measurement is essentially a makeshift. The datum sought as concentrating power is the urinary osmotic ceiling (maximum urine/plasma osmolar concentration ratio). It is the difficulty of measuring freezing point directly with a Beckman thermometer which has led to the use of the urinometer as a means of approximating osmolarity from density. This estimate is inherently inaccurate because of the variable proportion between osmolarity and density in inhomogeneous solutions, such as urine. Further, the urinometer, unless carefully used, may yield highly inaccurate readings.

A sensitive electrical device for registration of freezing point of a solution with a thermistor has been described,[5] and an apparatus using this principle made commercially available. This instrument is capable of measurements of osmotic concentration in the range 100 to 1,000 milliosmols (mOsm) per liter with an accuracy of the order of 1 per cent.

This note describes relationships between urinary specific gravity and osmolarity found in Addis concentration tests in patients with hypertensive and primary renal disease. Data from patients on unrestricted diets are compared with those from patients on sodium-restricted diets in order to demonstrate the effect of inhomogeneity of urinary solutes on this relationship. Lastly, observations from urine of patients on sodium restriction are used as a test of the intrinsic accuracy of urinometry in this laboratory.

PROCEDURE

Urines were collected during the last 12 hours of a 24-hour period of fluid deprivation. Specific gravity was measured with a certified hydrometer (range 1.000-1.070) and corrected to 15° C. and for proteinuria (by subtracting 0.003 for each 10 Gm. of protein per liter of urine). The urinary osmolarity was measured with the Fiske Associates Osmometer using 1 ml. samples of urine diluted with 1 ml. of distilled water.

The data (third and fourth decimal places of the specific gravity measurements and urinary osmolarity in mOsm per liter) were used in calculations of correlation coefficients, regression equations, and standard errors of the estimates,[6] having been grouped according to diet as unrestricted, 1 to 2 Gm. Na daily, 0.2 to 0.5 Gm. Na, and 0.2 to 2.0 Gm. Na.

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RESULTS

The data are summarized in Table I. The effect of dietary restriction of sodium is to increase the degree of the association between density of osmolarity, most of all in those patients whose urine contains the least sodium; the other effect of sodium restriction is to decrease urinary osmolarity per unit density. Both effects are predictable results of decreased urinary sodium and chloride content.

TABLE I. SUMMARY OF OBSERVATIONS ON URINARY SPECIFIC GRAVITY AND OSMOTIC CONCENTRATION IN CONCENTRATION TESTS

DIET	NUMBER OF SAMPLES	CORRELATION COEFFICIENT	STANDARD ERROR OF ESTIMATE
Unrestricted	54	0.89	±96
Restricted			
1-2 Gm. Na	16	0.96	±50
0.2-2.0 Gm. Na	42	0.89	±70
0.2-0.5 Gm. Na	26	0.90	±31

Correlation coefficients and standard errors of estimates indicating the association between urinary specific gravity and osmolarity (U_{mOsm}). The corresponding regression equations are as follows: unrestricted, Sp. Gr. = 1.022 (U_{mOsm}–873) + 22.7; restricted, 1–2 Gm. Na, Sp. Gr. = 1.023 (U_{mOsm}–758) + 21.6; 0.2–2.0 Gm. Na, Sp. Gr. = 1.030 (U_{mOsm}–730) + 21.7; 0.2–0.5 Gm. Na, Sp. Gr. = 1.041 (U_{mOsm}–712) + 21.8. The errors of estimate decrease with the Na content of the diet; in graphic representation, these corresponded well with the spread of data around lines drawn from the regression equations. The initial multipliers of the latter increase with decreasing dietary sodium, demonstrating the greater density per unit of osmotic concentration of salt-poor urine.

The scatter of data in urines of patients on strict (0.2 to 0.5 Gm. Na) diets presumably reflects largely the inherent error of urinometry, since the composition of urines in these patients is relatively homogeneous, and the measurement of osmotic concentration intrinsically accurate. This error is surprisingly small, corresponding to ± 0.00015 unit of specific gravity or, respectively, from 3 to 10 per cent of the urinary osmolarity in urines of high or low density.

While the last datum indicates a surprising relative accuracy of urinometry, the experience indicates the advantages of electrometric measurement of osmolarity. The datum thus provided from concentration tests is in effect the osmotic ceiling and, with little more inconvenience, simultaneous measurement of plasma osmolarity will yield the ultimate limiting factor in urinary concentration, namely, the osmotic U/P ratio. The measurement is intrinsically less variable than the estimate of concentrating power from specific gravity, since it is independent of the composition of the urine. A special advantage in hospital laboratories is the small volume of sample required. This, as the electrometric technique comes into routine use, should do away with the frequent and annoying reports of "N.S.Q."

SUMMARY

Urinary specific gravity and electrometrically measured osmolarity were compared in urine concentration tests. The tests were done in patients with renal disease, in some of whom dietary sodium was restricted. Predictably, the two functions were most closely associated and specific gravity higher per milliosmol in urine of low sodium chloride content in patients on sodium restriction. The data from urines of very low sodium content indicate that careful

urinometry is a relatively accurate procedure, but experience shows that electrometric measurement is practically, as well as theoretically, a more desirable routine procedure.

Dr. Sherman Watson, a visitor from the research laboratories of Sharp and Dohme, Inc., was very helpful in the compilation of these data.

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THE CULTIVATION OF TISSUES IN SMALL SEALABLE DISHES

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MOST tissue cultures when employed for the isolation or study of viral agents are maintained at the present time in glass tubes of various kinds. The advantages of the tube method, i.e., the direct growth of the cells on the glass wall, the economy of materials, and the ease of handling, are offset when the type of study requires in addition the detailed microscopic study of tissue cells in situ. Carrel's original method (Carrel, 1923), using flat, round flasks with a removable bottom opening, allowed tissues adherent to the bottom glass

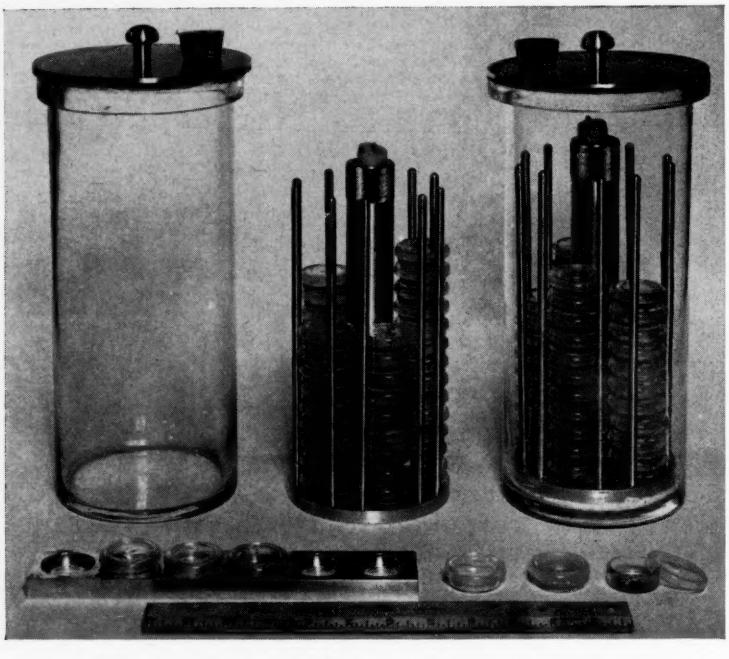

Fig. 1.—Tissue culture dishes, storage racks, and jars.

to be removed, stained, and studied at high magnifications in situ. But flasks of this type are expensive and more delicate and difficult to manipulate. Accordingly, a glass dish has been designed which combines the advantages of both methods of cultivation, and offers a relatively inexpensive, sturdy, easy-to-handle container which permits observations of living or stained cells on a flat surface

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with either direct or phase contrast illumination. Furthermore, such a container lends itself to stacking inside a chamber when a modified gaseous atmosphere is desired.

The dish is a modified Pyrex Petri plate having an inside diameter of 30 mm. and a depth of 12 mm.* The edge of the dish is ground, as is also a circular ring around the underside of the glass cover to permit a fluid-tight seal when the ground surfaces are lightly coated with a silicone grease.† The dishes are greased before sterilization, and after use may be cleaned by rinsing in

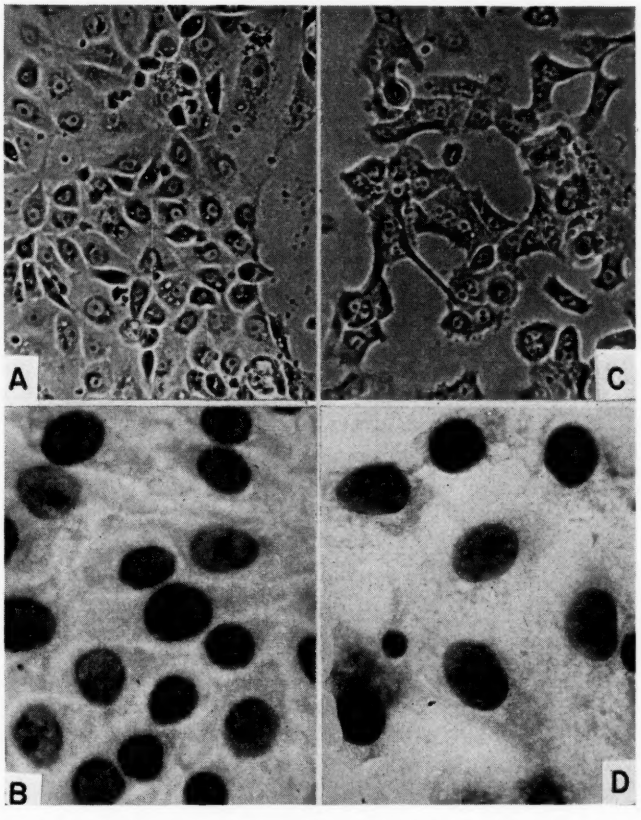

Fig. 2.—*A*, Monkey kidney cells: 12-day growth, living culture, phase contrast, ×210; reduced ⅓.
B, Monkey kidney cells: 5-day growth, stained with Giemsa, ×960; reduced ⅓.
C, HeLa cells: 3-day growth, living culture, phase contrast, ×227; reduced ⅓.
D, HeLa cells: 3-day growth, stained with Giemsa, ×576; reduced ⅓.

kerosene and washing with a metaphosphate detergent. The tissue dish holds 2.5 ml. of fluid, but in practice we have used 1.0 to 1.5 ml. of culture per dish. A metal strip containing recessed holes serves as a rack for the handling of these plates. To simplify the racking of numbers of dishes, a simple aluminum holder has been devised which resembles a poker chip rack. The center post

*These dishes are obtainable from Bellco Glass, Inc., Vineland, New Jersey.
†Dow Silicone stopcock grease.

is hollowed at the top to form a candleholder. In use, the rack of dishes is placed in a glass bacteriologic anaerobic jar, the candle is lit, and the jar lid replaced. The reduced oxygen tension obtained in this manner is sufficient to make gassing with a CO_2-air mixture unnecessary. Fig. 1 illustrates the entire assembly.

The sealed dish is fluid, but not gas-tight, and may be inverted on the microscope stage for short periods of observation of the living cells. Staining of cells is particularly simple since it is only necessary to fix and stain the tissue layer in the dishes. The flatness of the base and optical quality of the glass are adequate for observation of cell detail of stained preparations with oil immersion lenses.

Thus far, both monkey kidney and human epidermoid carcinoma tissues (strain HeLa) (Scherer and associates, 1953) have been maintained in a healthy state by these methods for 3 weeks or more. The trypsinized monkey kidney cells (Youngner, 1954) were grown and maintained thereafter in mixture No. 199 plus 2 per cent horse serum. The HeLa cells were grown in 60 per cent Hanks' balanced salt solution plus 40 per cent human serum and maintained thereafter in 85 per cent Hanks' balanced salt solution plus 10 per cent chicken serum and 5 per cent chick embryo ultrafiltrate. Photographs of such cultures by phase contrast and direct microscopy are shown in Fig. 2. The cells illustrated are of various ages (3 to 12 days) and show early and advanced stages of formation of tissue sheets.

In general, the viability and appearance of cells in these dishes have been superior to control batches of the same tissues cultivated in screw-cap tubes at the same time and in the same lots of culture fluids.

SUMMARY

A sealed glass dish together with an effective storage rack for use in the cultivation of tissues have been described. This culture dish permits luxuriant growth of certain tissues and facilitates detailed observation of cell morphology.

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A NEW COMPLEMENT FIXATION TEST FOR SYPHILIS

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THE introduction of another complement (C′) fixation test for syphilis to the many procedures already in use requires some justification. The C′ fixation test to be outlined represents a precise and carefully controlled procedure based on the results of modern research on C′ and C′ fixation. Yet it is a simple and practical method.

Most C′ fixation procedures for the serodiagnosis of syphilis, such as the Kolmer test, rely on the standardization of C′ in terms of the amount of C′ required to effect complete or "100 per cent" hemolysis. Greater precision, however, may be attained by the use of C′ standardized in terms of a 50 per cent hemolytic unit ($C'H_{50}$). Such a unit may be determined by noting the C′ amounts required to effect intermediate percentages of hemolysis. A diagram of the percentage responses against the logarithm of the C′ amounts leads to a symmetrical sigmoid curve.[1] Since the sigmoid curve is asymptotic to 0 per cent or 100 per cent, C′ assays based on 100 per cent hemolysis are arbitrary and subjective. The hemolytic curve may be converted to a straight line, moreover, by conversion of the percentages of hemolysis to the corresponding probit values.[2] The $C'H_{50}$ may then be determined accurately by calculation or visual inspection.

Osler and his colleagues[3, 4] have described an excellent, practical method for diagnostic C′ fixation procedures which uses a 50 per cent C′ unit and controls the various chemical and biologic factors known to influence the activity of C′. Their method features the use of a spectrophotometer. A spectrophotometer is an expensive instrument and may not be readily available in all serologic laboratories desiring to use the complement fixation method. We have modified the method of Osler and his colleagues so that a spectrophotometer is not required and have greatly simplified the calculation of the $C'H_{50}$ unit. Thus, neither instrumentation nor mathematics need deter the use of a precise standardization of C′. In addition, for the serodiagnosis of syphilis, the cardiolipin antigen and C′ amounts have been changed so that the procedure is set at a lower reactivity level. Finally, a two-tube test has been substituted for the four-tube procedure, resulting in a simplified method.

MATERIALS

1. Diluent: Isotonic Veronal buffer which contains optimal amounts of calcium and magnesium is used: 83.8 Gm. NaCl, 2.52 Gm. $NaHCO_3$, 3.00 Gm. sodium 5, 5-diethyl barbiturate, 4.60 Gm. 5, 5-diethyl barbituric acid, 1.2 Gm. $MgSO_4 \cdot 7H_2O$, 0.2 Gm. $CaCl_2 \cdot 2H_2O$.

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The last three components are dissolved in 500 ml. of hot distilled water and are added to the solution of the remaining components. The combined solutions are cooled, and distilled water added to 2,000 ml. One volume of this stock buffer is diluted as needed by the addition of 4 volumes of distilled water. The pH of the diluted buffer is about 7.4. It constitutes what is referred to as the *diluent*.

2. Sera and cerebrospinal fluids: Test sera are inactivated by heating at 56° C. for 30 minutes. Cerebrospinal fluids are tested without heating.

3. C′: Commercial, lyophilized guinea pig serum is used. The lyophilized serum is reconstituted to the original volume with the chilled solution supplied by the manufacturer.

4. Rabbit antisheep amboceptor (ASA): ASA, commercially prepared and preserved with an equal volume of glycerol, is used. A 1:100 dilution of the serum is stored at about -10° C. and used as a stock solution.

5. Sheep red cells: Sheep blood is preserved at about 4° C. in an equal volume of sterile, modified Alsever's solution. The Alsever's solution is composed of 2.05 per cent dextrose, 0.42 per cent sodium chloride, 0.8 per cent trisodium citrate, and 0.055 per cent citric acid. In our experience, sheep erythrocytes have been satisfactory for use for a period of six weeks after collection in this solution.

6. Antigen: In the test for syphilis, a stock alcoholic solution containing 0.0175 per cent cardiolipin, 0.0875 per cent lecithin, and 0.3 per cent cholesterol is used.

STANDARDIZATION OF REAGENTS

1. Sheep cell suspension: A 3 per cent suspension is prepared daily. The cells are washed three times and suspended in the diluent.

2. Estimation of the optimum quantity of ASA for use in the test: A series of dilutions of ASA are prepared, covering the expected range of activity of the ASA. Equal volumes (about 10 ml.) of the sheep cell suspension and of each individual dilution of the ASA are placed in separate beakers. The ASA is poured onto the sheep cells, and the mixture is poured back and forth at least three times. Ten minutes are allowed to elapse before the sensitized cell suspension is used.

Four milliliters of the diluent is added to 1.5 ml. of each of the sensitized cell suspensions, and then 2.0 ml. of a C′ dilution chosen to yield about 50 per cent hemolysis with an excess of ASA. The tubes are incubated at 37° C. for 60 minutes and read as in the C′ titration described later in Section 4. A point is reached in titrating most amboceptors beyond which additional amounts of ASA do not appreciably affect the hemolytic activity of C′.[5] Thus, when an appropriate dilution of ASA is used in C′ fixation tests, the additional contribution of natural amboceptor present in some human and animal sera should not enhance the hemolytic activity of C′.

A typical result obtained with one lot of amboceptor at this laboratory is shown in Fig. 1. The optimum concentration for this lot was 1/3,200.

3. Sensitization of the sheep cell suspension: The amboceptor is diluted in accordance with the determinations of the preceding section. This dilution is then used in all tests without further standardization. Sheep red cells are sensitized by pouring the diluted amboceptor on the cells, and the mixture is poured back and forth from one flask to another at least three times to mix thoroughly. After standing 10 minutes, the suspension is used in the C′ titration. It is stored at 5° C. and added to the tests of sera and cerebrospinal fluids following the fixation periods.

4. C′ titration: The appropriate C′ dilution must be determined by a preliminary titration. C′ is titrated daily for each test series. Table I summarizes the procedure for the C′ titration and gives a typical result as an illustrative example. The C′ amounts of the appropriate dilution are, to a close approximation, equally spaced on a logarithmic scale. As seen, large volumes (7.5 ml.) are used to increase the precision of the complement titration which is performed in Pyrex tubes (25 by 150 mm.). The tubes are placed in a water bath at 37° C. for 60 minutes. The contents of each tube in the titration are mixed thoroughly every ten minutes. After one hour, the tubes are removed from the

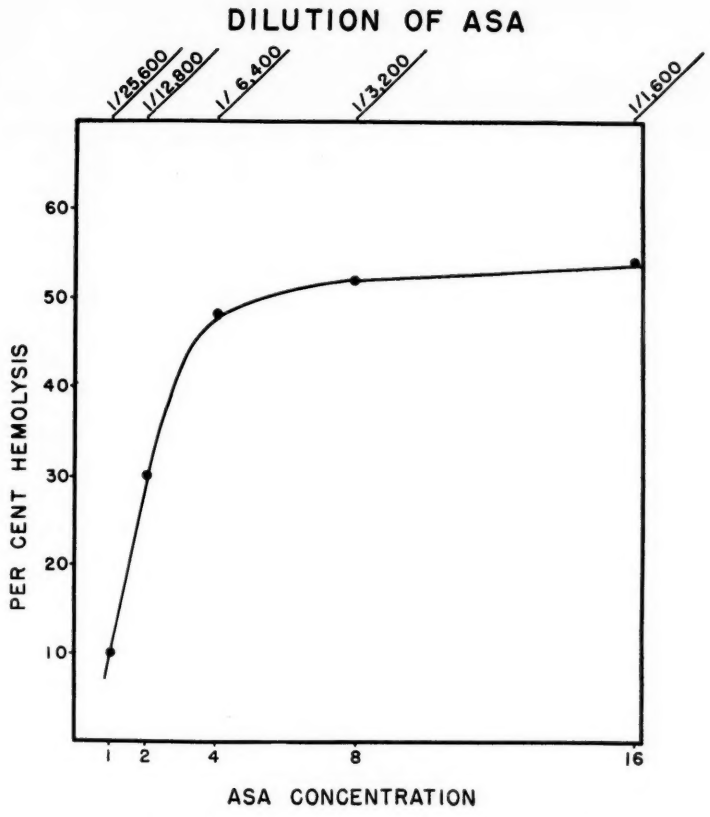

Fig. 1.—Per cent hemolysis vs. ASA concentration with a constant amount of C′.

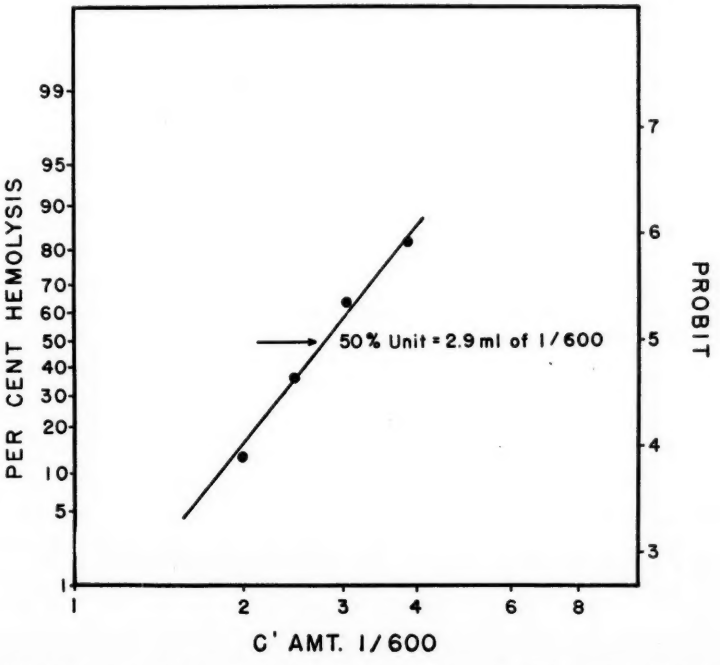

Fig. 2.—C′ titration per cent hemolysis on probit scale vs. the C′ amount diluted 1/600 on a log scale.

water bath and compared with a set of color standards prepared as indicated in Table II. A visual estimate of the percentage hemolysis is made. Tubes showing less than 70 per cent hemolysis are centrifuged, and the clear supernatant fluid is used in obtaining the percentage hemolysis. The results are illustrated graphically in Fig. 2.

TABLE I. C′ TITRATION

TUBE NO.	SENSITIZED SHEEP RBC (ML.)	DILUENT (ML.)	C′ DILUTION (ML.)	PER CENT HEMOLYSIS*
1	1.5	4.0	2.0	12.0
2	1.5	3.5	2.5	36.5
3	1.5	2.9	3.1	63.0
4	1.5	2.1	3.9	82.0
5	1.5	6.0	0.0	0.0

*Duplicate determinations are made. Results represent the mean of two estimated values and are given here merely as an example.

TABLE II. PREPARATION OF COLOR STANDARDS FOR THE C′ TITRATION

	PER CENT HEMOLYSIS										
	0	10	20	30	40	50	60	70	80	90	100
Oxyhemoglobin,* (ml.)	0.0	0.6	1.2	1.8	2.4	3.0	3.6	4.2	4.8	5.4	6.0
Sensitized cells (¼) (ml.)	6.0	5.4	4.8	4.2	3.6	3.0	2.4	1.8	1.2	0.6	0.0
C′ dilution, inactivated (ml.)	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5

*Oxyhemoglobin: Centrifuge 12 ml. of sensitized cells. Remove the supernate and lyse the sedimented cells with 45.6 ml. of water. Make solution isotonic by addition of 2.4 ml. of 17 per cent sodium chloride. Centrifuge standards from 0 to 60 per cent.

Without any conversion, the complement amount is plotted against the per cent hemolysis on logarithmic normal paper.* In essence, such paper automatically effects a conversion of the C′ amount to the logarithm of that amount and the per cent hemolysis to the probit of that value. No mathematical manipulation of the experimental results is thus required. The illustrative example may be plotted as indicated in Fig. 2. The best fitting line is drawn by visual estimate. Greater weight is given to points nearer the 50 per cent point. In the more than fifty titrations performed at this laboratory, the deviation of the points from a straight line has been extremely slight. The illustrative example is typical.

In the example, the complement was diluted 1/600, and 2.9 ml. of this dilution was equivalent to one $C'H_{50}$. The volume of the test is reduced by a factor of five, that is, from 7.5 ml. in the complement titration to 1.5 ml. in the test proper, with a proportional reduction in the number of sheep erythrocytes. Thus, 2.9 ml. of a 1:600 dilution is equivalent to 5 $C'H_{50}$ in the 1.5 ml. test procedure. Since 8 $C'H_{50}$ was found to give a reasonable level of reactivity, that amount was selected for our test procedure. The C′ dose is contained in a volume of 0.5 ml. The correct C′ dilution may be calculated by direct proportion:

$$2.9 \times 8/5 : 600 = 0.5 : X$$
$$X = 65$$

A 1:65 dilution of the C′ will contain, therefore, 8 $C'H_{50}$ in the 0.5 ml. test amount.

5. Antigen dilution: Four dilutions of cardiolipin antigen were selected for preliminary tests: 1:1,200, 1:600, 1:300, and 1:150. Sera with a high content of reagin reacted strongly with all four antigen dilutions. The 1:300 dilution proved optimum in most cases with weaker sera, and that dilution was finally selected. For cerebrospinal fluids, the

*This may be either logarithmic-normal deviate paper (such as Paper 32: 376, Codex Book Co., Norwood, Mass.) or logarithmic-probit paper (such as Keuffel and Esser Paper No. 358-22).

antigen dilution was 1:60. The amount of antigen added was equal, however, to the antigen amount in the serum test since a proportionate reduction was made in the volume of antigen dilution used. This adjustment is required because of the large test volume of cerebrospinal fluid.

The antigen is diluted by measuring the stock antigen and diluent into separate beakers. The diluent is then poured on the antigen and the mixture poured back and forth several times.

TEST PROCEDURE

The tests were performed according to the outline in Table III. The test proper consists of one tube containing serum or cerebrospinal fluid with complement and antigen. The serum or cerebrospinal fluid control tube without antigen serves as an anticomplementary control for each specimen. The complement control tubes serve to check the uniformity of the hemolytic activity of the complement after the fixation period. In most instances, the 0.10 ml. amount has given about 25 per cent hemolysis, and the 0.15 ml. amount about 90 per cent hemolysis. Comparison of the results of the complement and antigen controls indicates the anticomplementary activity of the antigen. The sensitized sheep red blood cells control serves as a fragility test for the red cells.

TABLE III. 50 PER CENT COMPLEMENT FIXATION TEST FOR SYPHILIS WITH SERA AND CEREBROSPINAL FLUIDS (CSF)

A. Sera

	SERUM (ML.)	DILUENT (ML.)	8 $C'H_{50}$ PER 0.5 ML. (ML.)	ANTIGEN, 1/300 (ML.)
Serum test	0.10	0.10	0.50	0.50
Control	0.10	0.60	0.50	0.0
C′ controls	0.0	1.10	0.10	0.0
	0.0	1.05	0.15	0.0
Antigen controls	0.0	0.60	0.10	0.50
	0.0	0.55	0.15	0.50
Sensitized RBC control	0.0	1.20	0.0	0.0

B. CSF

	CSF (ML.)	DILUENT (ML.)	8 $C'H_{50}$ PER 0.5 ML. (ML.)	ANTIGEN, 1/60 (ML.)
CSF test	0.50	0.10	0.50	0.10
Control	0.50	0.20	0.50	0.0

The tubes are incubated for 17 hours at 4° C. plus 20 minutes at 37° C. Then 0.3 ml. of sensitized sheep RBC are added to all tubes. The tubes are then reincubated at 37° C. for 30 minutes and read.

READING AND EVALUATION OF TESTS

The reaction in the various test and control tubes is estimated visually by comparison with a set of color standards. The color standards are prepared as indicated in Table IV from a portion of the sensitized cells used for the test.

No hemolysis is designated as 4+, 25 per cent hemolysis as 3+, 50 per cent hemolysis as 2+, 75 per cent hemolysis as 1+, and 100 per cent hemolysis as negative. In evaluating test results, sera or cerebrospinal fluids giving at least 1+ to 3+ reaction are considered doubtful, and those giving a 4+ reaction are considered positive if the respective control tubes give 100 per cent hemolysis, indicating, therefore, the absence of anticomplementary activity. When the serum or cerebrospinal fluid control tubes indicate anticomplementary action, the tests are reported as doubtful if the difference between the test and control tube is equal to at least 50 per cent. However, if the test tube of an anticomplementary serum shows no hemolysis, the reagin content of that serum may be great, and the test should be repeated on a serum dilution estimated to avoid a manifest anticomplementary effect.

TABLE IV. COLOR STANDARDS FOR READING TEST RESULTS

	PER CENT HEMOLYSIS				
	0	25	50	75	100
Oxyhemoglobin solution,* (ml.)	0.0	0.375	0.75	1.125	1.5
Sensitized cells (1/5), (ml.)	1.5	1.125	0.75	0.375	0.0

*Centrifuge 3 ml. of sensitized cells. Remove the supernate completely and lyse the sedimented cells with 14.25 ml. of distilled water. Restore to isotonicity with 0.75 ml. of 17 per cent sodium chloride.

RESULTS

All the serum specimens tested by the procedure outlined, which will be designated as the 50 per cent unit cardiolipin complement fixation test, were also tested routinely by the cardiolipin microflocculation test of the U. S. Army and Air Force. Comparative results are given in Table V. There was complete agreement between the two tests in 697 or 87 per cent of the sera tested and partial agreement (negative and doubtful, or doubtful and positive) in 83 or 10 per cent. Only 19, or less than 2.5 per cent, of the sera showed complete disagreement. An evaluation of all patients from whom these specimens were obtained was not available. In approximately four out of five cases where partial or complete disagreement did exist, a higher level of reactivity is seen in the cardiolipin microflocculation test. This is a reflection of the fact that the cardiolipin microflocculation test is deliberately set at a high reactivity level since it is used as a screening procedure in the medical installations of the U. S. Army and Air Force.

TABLE V. COMPARISON OF THE 50 PER CENT CARDIOLIPIN COMPLEMENT FIXATION TEST (50 PER CENT CCF) AND THE CARDIOLIPIN MICROFLOCCULATION TEST OF THE U. S. ARMY AND AIR FORCE (CMF) WITH SERA

	NO.	PER CENT
Agreement in both tests		
Negative	581	
Doubtful	10	
Positive	106	
Total	697	87.2
Greater reactivity in CMF		
Pos. CMF, Dbt. 50% CCF	15	
Dbt. CMF, Neg. 50% CCF	50	
Pos. CMF, Neg. 50% CCF	16	
Total	81	10.1
Greater reactivity in 50% CCF		
Pos. 50% CCF, Dbt. CMF	12	
Dbt. 50% CCF, Neg. CMF	6	
Pos. 50% CCF, Neg. CMF	3	
Total	21	2.6

Some of the test sera were also compared to the cardiolipin complement fixation test of the U. S. Army and Air Force. The latter procedure is essentially a Kolmer technique using a cardiolipin antigen. Comparative results are indicated in Table VI. Where disagreement occurred, greater reactivity was seen in the test procedure described in this study. Noteworthy is the fact that no specimen which gave a positive reaction in the standard Army and Air Force test failed to react in our procedure. One hundred and eighty-four

TABLE VI. COMPARISON OF THE 50 PER CENT CARDIOLIPIN COMPLEMENT FIXATION TEST (50 PER CENT CCF) AND THE CARDIOLIPIN COMPLEMENT FIXATION TEST OF THE U. S. ARMY (ARMY CCF) WITH SERA

	NO.	PER CENT
Agreement in both tests		
Negative	105	
Doubtful	8	
Positive	93	
Total	206	80.2
Greater reactivity in Army CCF		
Pos. Army CCF, Dbt. 50% CCF	6	
Dbt. Army CCF, Neg. 50% CCF	5	
Pos. Army CCF, Neg. 50% CCF	0	
Total	11	4.3
Greater reactivity in 50% CCF		
Pos. 50% CCF, Dbt. Army CCF	26	
Dbt. 50% CCF, Neg. Army CCF	12	
Pos. 50% CCF, Neg. Army CCF	2	
Total	40	15.5

cerebrospinal fluids were also subjected to both complement fixation procedures. The results indicated in Table VII show the same general relations as the tests on sera.

TABLE VII. COMPARISON OF THE 50 PER CENT CARDIOLIPIN COMPLEMENT FIXATION TEST (50 PER CENT CCF) AND THE CARDIOLIPIN COMPLEMENT FIXATION TEST OF THE U. S. ARMY AND AIR FORCE (ARMY CCF) WITH CEREBROSPINAL FLUIDS

	NO.	PER CENT
Agreement in both tests		
Negative	160	
Doubtful	0	
Positive	17	
Total	177	96.2
Greater reactivity in Army CCF		
Pos. Army CCF, Dbt. 50% CCF	1	
Dbt. Army CCF, Neg. 50% CCF	1	
Pos. Army CCF, Neg. 50% CCF	0	
Total	2	1.1
Greater reactivity in 50% CCF		
Pos. 50% CCF, Dbt. Army CCF	1	
Dbt. 50% CCF, Neg. Army CCF	1	
Pos. 50% CCF, Neg. Army CCF	3	
Total	5	2.7

The greater reactivity of the procedure described in this study compared to the cardiolipin Kolmer technique resulted in a test of greater sensitivity in the detection of clinical and latent syphilis but of lower specificity. The increasing availability of the *Treponema pallidum* immobilization test, however, should warrant the use of reagin tests of greater reactivity.

The precision of the C′ titration in the 50 per cent unit cardiolipin complement fixation test provided a test of great reproducibility. Different portions of a single serum tested on 25 different days gave a 3+ reaction on 18 occasions and a 2+ or 4+ reaction the 7 other times.

SUMMARY

A two-tube complement fixation test for syphilis embodying a 50 per cent C′ unit and a cardiolipin antigen has been described. It is set at a reactivity level intermediate between the two serologic procedures in use by the U. S. Army and Air Force. Because of the reasonable reactivity level, however, the test described would probably serve well as a screening or diagnostic tool.

This communication presents a procedure which is easily performed by any competent laboratory technician and does not require any expensive equipment. Although rigorously controlled and based on the results of modern research on C′ and C′ fixation, it is a practical, simple, and reproducible procedure.

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A PERFUSION TECHNIQUE FOR THE STUDY OF LIVER METABOLISM

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THE study of protein metabolism is important to the understanding of normal physiology and is of the utmost significance in investigations which deal with the rehabilitation of severely burned or traumatized individuals who must resynthesize large amounts of lean body mass. Liver perfusion is a useful method for the study of the hepatic function in the nutritional status of the organism, and, more particularly, of the relationship of the liver to protein metabolism. While other methods for organ perfusion have been described previously,[1-4] none have been widely used because of technical complexity. A simplified perfusion technique has been devised which, it is believed, will be a valuable tool to other investigators in studies of the biochemistry of the liver and other organs.

Our technique of perfusing rabbit livers is described. Data are given from a typical experiment to illustrate the measurement of the metabolic processes of rabbit livers under these experimental conditions. Studies on rates of protein synthesis and the influence of endocrine substances on these rates are reported elsewhere.[5]

METHODS AND MATERIAL

Animals.—Nonfasting, white New Zealand rabbits, weighing about 4 kg., were used in this study. A standard stock diet of pellets with fresh vegetable supplements was maintained throughout the study.

Blood.—On the afternoon preceding the perfusion of a liver, blood was collected from donor animals under light ether anesthesia. The animal was placed on its back, and the peritoneal cavity opened by a midline incision. The aorta was isolated below the renal arteries; and a 15-gauge polyethylene cannula was inserted. This cannula was connected to an evacuated, round-bottomed liter flask containing glass beads. The formation of large clots was avoided by swirling the flask as the blood was collected. This resulted in effective defibrination. Only enough heparin was used in each case to avoid clotting in the cannula. Seven hundred to 750 ml. of blood was collected (100 to 150 ml. from each animal) by exsanguination. This procedure proved more effective than drawing blood by cardiac puncture since pericardial and epicardial fibrosis following the first puncture made second and third punctures difficult. After the addition of 25 mg. Aureomycin, the perfusate was allowed to stand with intermittent shaking for about four hours at room temperature. Four milliliters of heparin (1,000 units per milliliter) was then added, and the blood kept at 4° C. overnight. The following morning the blood was brought to room temperature and the clots removed by filtering through sterile cotton sponges.

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Perfusate.—The α-amino acid solution was prepared by dissolving 17.75 Gm. of an α-amino acid mixture and 3.5 Gm. of NaCl in 500 ml. water. This solution was then forced through a Seitz filter. The α-amino acid mixture contained the essential and some nonessential acids (see Appendix). The pH of this mixture was adjusted to 7.45 with 1 N NaOH, and 500 ml. was then added to 500 ml. of the defibrinated blood. The pH was again checked, and the resultant pH was invariably 7.45. Four milligrams of glycine-2-C^{14} with a specific activity of 1.45 mc. per millimole and a radiopurity of >0.99 per cent,* was added to the perfusate and the latter placed in the reservoir of the apparatus. The pump was started, and the perfusate circulated until its temperature reached 37.5° C.

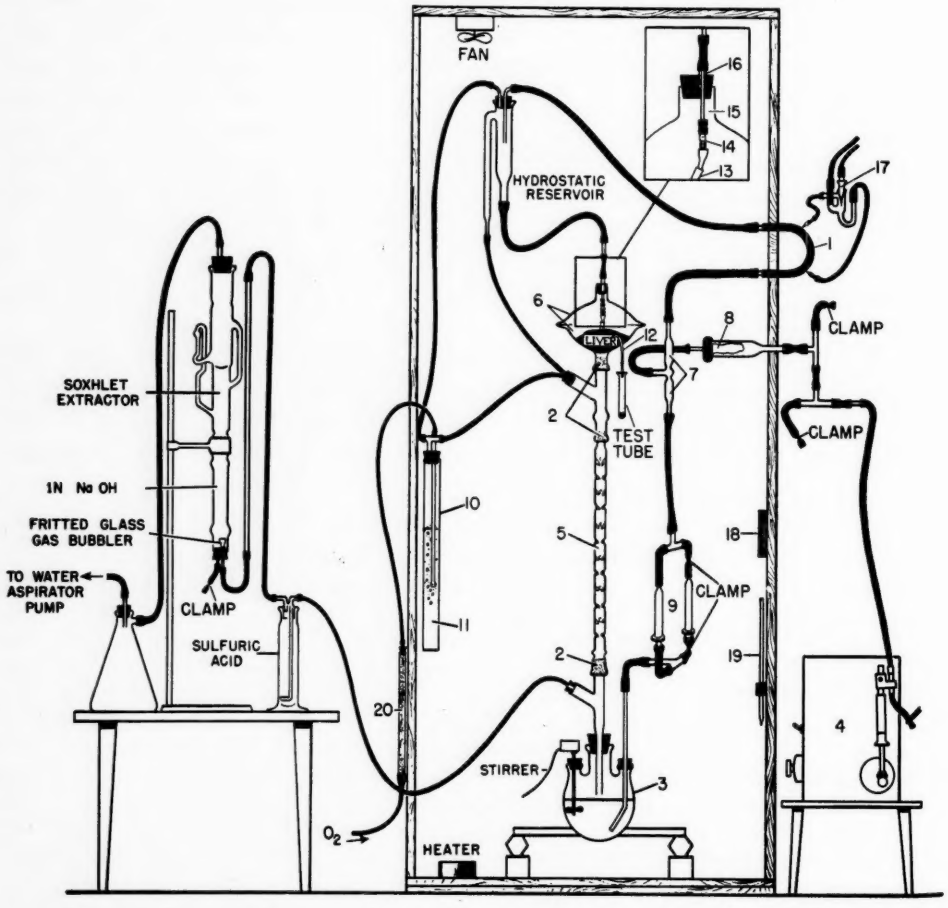

Fig. 1.—Liver perfusion apparatus. *1*, Thick-walled rubber tubing; *2*, ground-glass joints; *3*, three-neck liter distillation flask; *4*, automatic pipetting machine; *5*, Vigreaux distilling column; *6*, tops of desiccators; *7*, polished glass valves; *8*, finger of rubber glove; *9*, filters (Baxter); *10*, gas humidifier; *11*, saline; *12*, bile duct cannula; *13*, portal vein; *14*, Luer Lok fitting; *15*, glass tube 4 mm. outside diameter; *16*, glass tube 6 mm. inside diameter; *17*, pH flow electrodes; *18*, thermostat; *19*, thermometer; *20*, ascarite tube.

Perfusion Apparatus.—This apparatus is illustrated diagrammatically in Fig. 1. Essentially, the apparatus consists of an organ chamber where the liver is held in place; an oxygenating column; a reservoir for the bulk of the perfusate; a circulating pump; and a hydrostatic reservoir which supplies a constant flow of perfusate to the liver.

*Synthesized by Isotopes Specialties Company.

The organ chamber consists of two desiccator tops [*6*] placed with the ground surfaces opposing each other. These surfaces are lubricated to provide an airtight seal. The upper piece is hemispherical with an open top. A rubber stopper is inserted into this opening. As illustrated, a glass tube of 4 mm. outside diameter [*15*] is inserted through a tube of 6 mm. inside diameter [*16*]. This provides a tube of adjustable length for easy and quick regulation of the height of this tube to provide an anatomical position for the portal vein cannula [*13*]. A rubber collar is placed over the juncture of these two glass tubes ensuring an airtight seal. One member of a Luer Lok adapter [*14*] is attached to the lower end of the movable tube and the opposite member is adapted to the cannula in the portal vein [*13*]. The bottom piece of the organ chamber [*6*] is a shallow top from a vacuum desiccator. This provides a support for the liver which allows the lobes to lie freely about the vena cava and the portal vein without obstruction.

The oxygenation system consists of a fractionation column [*5*] with ground-glass joints [*2*]. A T-joint with ground-glass fittings is in place just below the organ chamber, and is connected to the Vigreaux fractionating column. An adapter with a side arm is joined to the fractionating column at its lower end, and then is inserted through a Fenwal cap which fits over the center neck of the reservoir. The diameter of this adapter must be 15 mm. or greater, since a smaller orifice causes constriction in the flow of perfusate into the reservoir. Under these circumstances the perfusate will accumulate in the fractionating column and be blown into the CO_2 exhaust. The oxygen is introduced into the system through a humidifier [10] filled with saline, after which the gas traverses the oxygenation column. It is then bubbled through a 20 per cent sulfuric acid solution for the removal of moisture and then through a solution of 1 N NaOH. The gas is drawn through the system by a water aspirator pump. Oxygen flow is regulated by a diaphragm valve in order to maintain a steady pressure. A manometer is placed in the inlet O_2 tube and slightly positive pressures are maintained (0.5 cm. Hg). Slight positive pressures offer greater convenience in balancing the inflow and outflow of gases. Negative pressures are difficult to regulate, and, if sufficiently low, cause foaming. Foaming and accumulation of perfusate in the oxygenating column [*5*] result in clotting, loss through the CO_2 exhaust, and insufficient oxygenation. When CO_2 production was measured, 100 per cent O_2 was used; and it was passed through an ascarite tube for removal of any possible CO_2 contamination.

The perfusion reservoir is a three-neck liter flask [*3*]. A stirrer is placed in one side neck, and the uptake tube to the circulating pump in the other. Sedimentation of red cells and increased fluctuations in the plasma protein concentrations were evident in experiments not employing a stirrer. A small amount of foam may form as the blood falls into the reservoir, but this is not troublesome under the described conditions. Air space must be allowed between the tube entering the reservoir and the surface of the perfusate to prevent the blood from backing up into the oxygenation column [*5*].

The circulating pump is essentially a lift pump. A pair of highly polished glass valves [*7*] are actuated by a pulsating tube. The pulsating tube [*8*] is a finger cot which is caused to pulsate by the action of an automatic pipetting machine [*4*] whose valves have been removed to obtain a reciprocating action. The pump can deliver from 20 to 300 ml. per minute and the flow through the liver was approximately 150 ml. per minute. By this means, circulation of the perfusate is accomplished with minimal red cell lysis. Measurable hemolysis, however, does result over the 12-hour period. The blood is drawn up through a nylon filter and into the hydrostatic reservoir.

The hydrostatic reservoir was made in this laboratory and is simply a 40 by 75 mm. Pyrex tube which has a side arm of 15 mm. and is reduced at the bottom to 6 mm. diameter. The hydrostatic pressure was approximately 30 cm. of perfusate. The container is sufficiently large so that the rate of flow to the liver does not change the hydrostatic pressure appreciably between the strokes of the pump. The reservoir has an air vent connected to the aeration system which equalizes the pressure above the reservoir; and the side arm is of a large diameter (15 mm.), allowing a noncontinuous overflow when the liver is not utilizing all of the perfusate delivered by the pump.

The apparatus is maintained at 37.5° C. ± 0.5° in a thermostatically controlled box. The box has Plexiglas on the front and one side, with a sampling tube of gum rubber [*1*] brought out through one side. The pipetting machine is also outside the box, permitting regulation of the perfusion pump and sampling without entrance into the box. Hydrogen ion flow electrodes [*17*] are connected to the system through a shunt in the sampling tube, as illustrated; and the pH of the perfusate can be observed periodically. The pH meter is a Beckman Model H-2 with an internal reference standard. After the perfusate became 37.5° C., the pH meter was standardized to the measured pH of the blood.

Sterility.—Every possible precaution was taken to avoid bacterial contamination. The entire apparatus, with the exception of the electrodes, was thoroughly cleaned and autoclaved. The electrodes were washed with alcohol and subsequently rinsed with sterile saline. The addition of 25 mg. Aureomycin at 4-hour intervals has been an effective method for the prevention and/or elimination of bacterial growth. All samples were obtained with sterile syringes after sponging the sampling tube with tincture of iodine. When cultures for bacteriologic studies were drawn, the needle and culture tubes were flamed in order to prevent false positive cultures.

An appendix is provided which lists the special items used in this apparatus and their dimensions.

Hepatectomy.—The rabbit was anesthetized with intramuscular paraldehyde, and the abdomen opened through a midline incision. The gastrohepatic and triangular ligaments were divided; and the inferior vena cava was isolated, ligated, and divided between the liver and right kidney. The hepatic artery was isolated, ligated, and divided. The common bile duct and lymphatics were divided; and the bile duct was then cannulated with an 18-gauge polyethylene cannula. The portal vein is isolated proximally from the liver for about 2 inches. All subsidiary vessels are ligated and divided. The portal vein was then ligated and cannulated with a polyethylene tube of about 3 mm. outside diameter. Ten milliliters of 1/10 dilution of heparin were rapidly injected through the cannula into the liver. The thorax was then opened, and the thoracic inferior vena cava isolated. The diaphragm was incised about the vena cava, leaving a small cuff. All diaphragmatic veins were carefully ligated. The right and left diaphragmatic crura were then clamped, divided, and ligated; and a hemostat was placed on the vena cava about 1 inch above the diaphragm. This vessel was then divided, and the liver lifted free from the animal. The vena cava was intubated with a cannula of the same size as used in the portal vein, and the liver was weighed. The organ was then carried to the perfusion apparatus and placed on gauze sponges in the organ chamber by placing the vena cava cannula through the opening of the lower desiccator top. The portal vein cannula with its female Luer Lok adapter was connected to the male Luer Lok in the upper half of the organ chamber. The blood was then allowed to flow through the liver by unclamping the tube from the hydrostatic reservoir, and the pump was started. In order to prevent air embolization to the liver, the hydrostatic reservoir had been previously filled to exclude all air from the tube to the portal vein cannula. The position of the liver was adjusted to ensure unobstructed ingress and egress to and from the lobes. If the liver was properly arranged, blood appeared in the vena cava cannula almost instantaneously. Otherwise, there was a lag in the appearance time of the blood, and marked distention of the portal vein and/or lobar turgidity appeared. The liver having been properly placed, the common bile duct cannula was led out of the organ chamber through a hole (made by means of a dental drill) in the side of the lower desiccator top, and the bile collected in a calibrated test tube.

Protocol for Sampling.—Zero-hour samples were drawn after several circulations of the blood. Hourly samples were drawn from chemical analysis and for C^{14} assay in those studies utilizing this isotope. Bacteriologic cultures were drawn at 4-hour intervals, and 4,000 units of heparin and 25 mg. Aureomycin were injected every fourth hour. Samples were placed in tubes containing approximately 10 mg. NaF, covered with "Parafilm" and stored at 4° C. until the following day. A sample of whole perfusate was taken from the zero- and 12-hour samples for hematocrits. Whole perfusate samples were lyzed by freezing and thawing

for the determination of C^{14} recovery. The zero-hour sample was taken as the initial concentration of C^{14} in the perfusate. The red cells of the samples were spun down, the plasma was decanted, and the latter was then analyzed for the various factors under study.

Chemical Analysis.—Expired CO_2 was absorbed in a known volume of 1 N NaOH. An excess of saturated $BaCl_2$ was added to an aliquot of this solution and the CO_2 precipitated as $BaCO_3$. The residual NaOH was then titrated against a standard HCl solution. Reagent blanks were carried out in the same manner to assure an appropriate correction for CO_2 contamination. The reducing sugars were determined by the Somogyi[6] modification of the Folin-Wu method[7]; and the fructose concentrations were determined by the Roe resorcinol method.[8] Urea nitrogen was measured by the urease method of Gentzkow and Masen[9]; and the α-amino acid nitrogen by the Fister[10] modification of the Kober-Sugiura reaction.[11] Nonprotein nitrogen was determined in TCA filtrates of the plasma by micro-Kjeldahl digestion, steam distillation, and titrimetric determination. The samples were digested by the Ma and Zuazaga method,[12] transferred to the steam distillation apparatus, distilled, and ammonia was collected in 2 per cent boric acid. This was then titrated with a standard HCl solution. Appropriate dilutions were made to obtain concentrations of ammonia equivalent to 1 to 5 ml. of 0.01 N HCl. Total nitrogen was carried out on appropriate dilutions of the plasma. The digestion, distillation, and titration were carried out in the same manner as with nonprotein nitrogen.

Hemoglobin concentrations were determined by measuring the color density of acid hematin. A standard curve with the optical density of acid hematin plotted against the concentration of hemoglobin was prepared. The gram per cent of hemoglobin was divided by 6.14 to obtain gram per cent nitrogen. This procedure is described elsewhere.[5] The total nitrogen values were corrected for hemoglobin nitrogen. The protein nitrogen was then calculated as the difference between nonprotein nitrogen and the corrected total nitrogen.

Bromsulphalein was determined by the method of Dragstedt and Mills.[13]

C^{14} Assay.—The C^{14} was counted in a gas flow open window counter. The methods of C^{14} assay in various fractions of the liver perfusate and in the CO_2 are described[5] in a subsequent paper.

Histologic Preparations.—Histologic preparations were made of formalin-fixed specimens from various areas of the liver. Hematoxylin and eosin stains were made in order to observe the general histology of the liver; and Schiff's[14] stain was used to observe the histologic distribution of glycogen.

RESULTS

Microscopic Examination.—The histologic appearance of the livers showed slight to moderate edema and some evidence of red cell sludging in the portal sinusoids. No bacterial emboli were seen. Hematoxylin and eosin stained tissue slices of a perfused liver and a normal rabbit liver are pictured in Figs. 2 and 3, respectively. Tissues stained with Schiff's stain[14] (not shown here) show a decrease in glycogen of the perfused livers as compared to normal liver sections. This difference existed in the chemical analysis[5] of the livers.

Chemical Changes.—The values reported in Table I and illustrated in Fig. 4 are taken from one experiment in order to give an indication of the activity of the perfused liver. These results clearly indicate the presence of metabolically active tissue. There are significant changes in the urea nitrogen, α-amino acids, glucose, and fructose. Glycogenolysis is apparent from the release of glucose to the plasma. The glucose concentration in some experiments rose to values 2 to 3 times higher than those reported here. There was no significant trend in the chemically measured plasma protein concentration change; however, there is a definite incorporation of C^{14} from glycine-2-C^{14} into protein which steadily increases with time of perfusion.

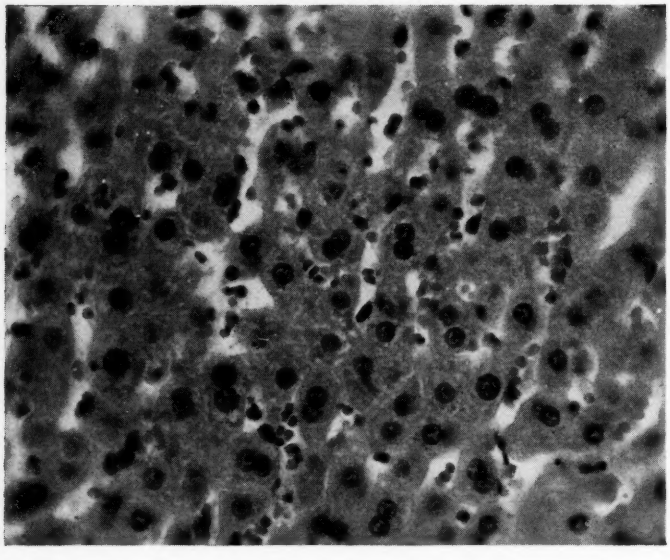

Fig. 2.—Section of a liver perfused for twelve hours under our experimental conditions. Major difference from normal discernible is the greater numbers of red blood cells visible in the sinusoids. (Hematoxylin and eosin, ×475; reduced ¼.)

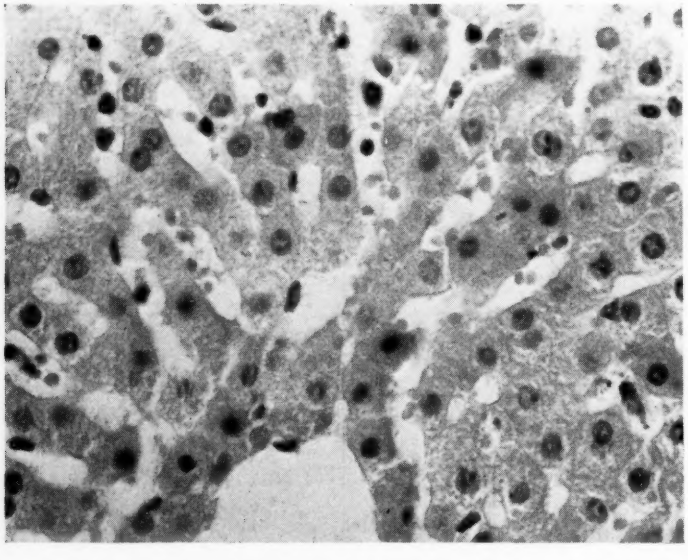

Fig. 3.—Section of a normal rabbit liver obtained immediately following death by a blow on head. (Hematoxylin and eosin, ×475; reduced ¼.)

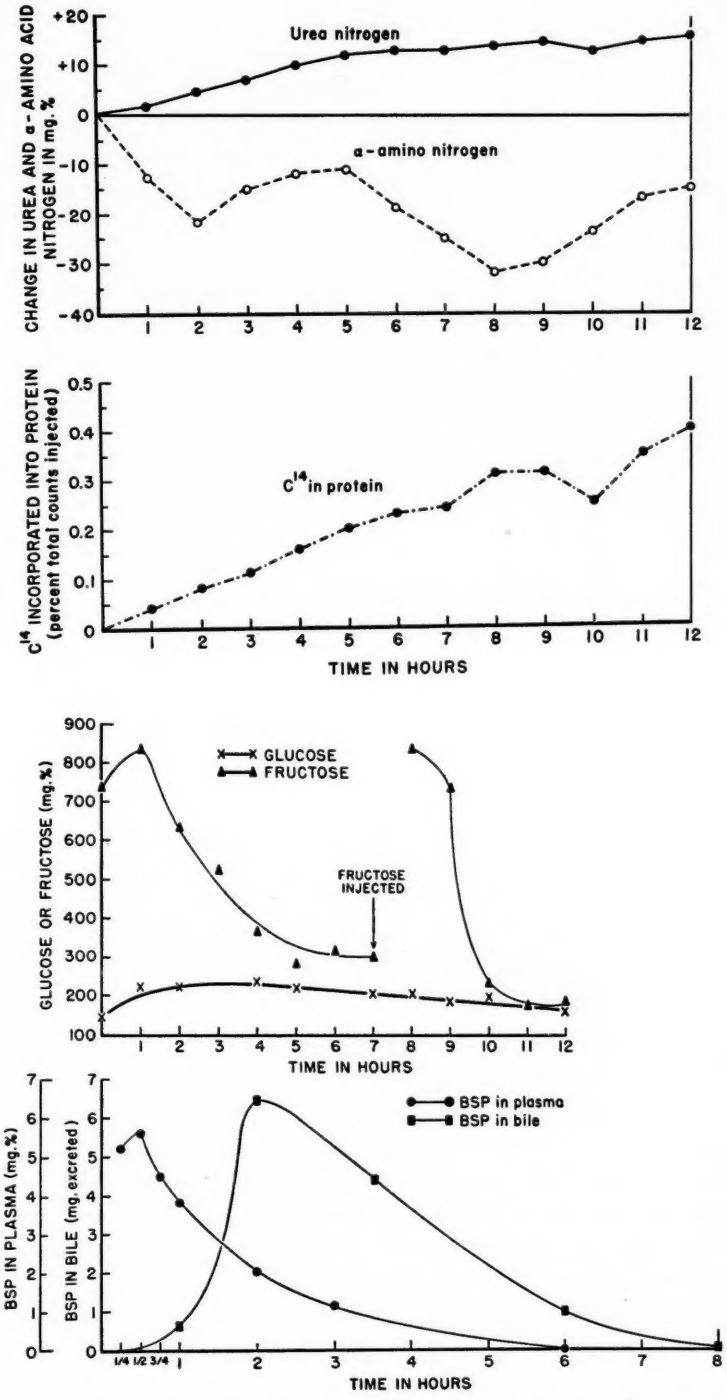

Fig. 4.—Changes in perfusate constituents during a typical liver perfusion experiment.

TABLE I

TIME	PROTEIN (GM. %)	C^{14} IN PROTEIN (% TOTAL)	α-AMINO NITROGEN (MG. %)	UREA NITROGEN (MG. %)	GLUCOSE (MG. %)	FRUCTOSE (MG. %)	BSP IN PLASMA (MG. %)	BSP* IN BILE (MG. EXCRETED)	BILE FLOW (ML. EXCRETED)	PH	CO_2 (ML./GM./HR.)	C^{14} IN CO_2 (% TOTAL)
0	2.35	0	179	5.1	144	739	—	—	—	7.45		
(min.)												
15	—	—	—	—	—	—	5.2	—	—	—		
30	—	—	—	—	—	—	5.6	—	—	—		
45	—	—	—	—	—	—	4.5	—	—	—		
60	2.52	0.4	167	6.4	222	834	3.8	0.61	0.3	7.45		
(hours)												
2	2.37	0.8	158	9.3	225	634	1.6	6.44	1.4	7.45		
3	2.39	0.11	165	12.0	—	528	1.1	4.34	0.9	—		
4	2.53	0.16	168	15	233	370	—	—	—	—	0.90	0.48
5	2.56	0.20	169	17	220	285	—	—	—	—		
6	2.58	0.23	161	18	—	317	0.0	0.94	0.8	7.45		
7	2.27	0.24	155	18	206	301	—	—	—	—		
8	2.36	0.31	148	19	204	840	0.0	0.09	0.5	—	0.60	0.49
9	2.40	0.31	150	20	184	734	—	—	0.4	7.45		
10	2.36	0.25	156	18	194	230	—	—	—	7.48		
11	2.37	0.35	163	20	172	172	—	—	—	7.50		
12	2.54	0.40	165	21	156	185	—	—	0.5	7.48	0.49	0.34

*Twenty per cent of the injected BSP was excreted in the bile.

Carbon dioxide production ranged from 0.4 to 1.0 ml. CO_2 per gram per hour, and the formation of $C^{14}O_2$ was 0.3 to 1.0 per cent of the total injected activity. The appearance of C^{14} in CO_2 suggests metabolic breakdown of the glycine-2-C^{14}.

The pH was measured throughout the run and remained quite constant. In the rare experiments grossly contaminated with bacteria, the pH dropped continuously to a final level of about 6.85. The removal of BSP from the perfusate by the livers has been tested and the values are given in Table I. Bile formation is continuous, ranging from 0.3 ml. bile flow per hour in this experiment to 3 ml. per hour in other experiments.

Table II is a summary of the counts per minute from the C^{14} in various fractions of the perfusate and the liver. The recovery value in this particular experiment was 103 per cent. Recovery percentages ranged from 95 to 105 per cent. This variation was presumably due to inaccuracies in the estimation of the volume of fluid present in the liver before the perfusion is started. Table II shows an uptake of C^{14} into the tissue fluids of the red cells and of the liver. The fraction of tissue fluid activity representing actual metabolic activity has not been ascertained.

TABLE II

FRACTION ASSAYED	PER CENT OF COUNTS
Liver protein	0.86
Plasma protein	0.40
Perfusate (whole blood)	90.65
Liver fluids (protein free)	9.80
$C^{14}O_2$	1.31
Total	103.02

Bacteriologic studies were made on all experiments. Most experiments were completely free of contamination, or only a few colonies were noted after 24 hours' incubation. In those experiments which had considerable contamination, the results were discarded in the evaluation of data. Microscopic observations of the liver on the uncontaminated or slightly contaminated experiments fail to show any bacteria in the tissue.

DISCUSSION

The greatest advantage of this apparatus is in the accessibility of the components. Most of the parts, such as the desiccator tops, reservoir, and fractionating column are readily available in any laboratory. The glass valves, pipetting machine, and pH meter can be purchased from scientific instrument companies. The hydrostatic reservoir was made here but can be easily duplicated. The apparatus is simple, allowing easy dismantling for autoclaving and rapid reassembly. The pump system is dependable and reasonably economical. The features of the apparatus described offer a somewhat standardized instrument which can be easily duplicated by other groups.

Many methodological difficulties were encountered in setting up this technique. These matters have been described above, but it is well to review some of these problems. Adjustment of the pressure in the aeration system is

important. This was stabilized by use of a manometer. For experiments in which CO_2 is not measured, the outflow gas can escape into the air freely and no pressure difficulties are encountered. Clotting was initially a problem, but this was eliminated by defibrination of the blood, and by the subsequent additions of adequate amounts of heparin.

Particular care must be exercised in arranging the liver in the organ chamber to avoid obstruction of the flow through the liver. Partial outflow obstruction causes turgidity of the liver, and obstruction of flow to individual lobes results in unperfused areas which become dark. These situations can be recognized and corrected promptly with experience.

Other difficulties involve the proper cannulation of the veins and bile duct. The cannulas were inserted just far enough into the vessels so that they could be securely tied. Too great a depth of insertion will cause obstruction of the portal ramification to one or more lobes. The cannulas were carefully arranged to rest in an anatomical position during perfusion. Clotting in the liver was prevented by the immediate injection of 10 ml. of a 1/10 dilution of heparin into the liver after the flow through the liver was stopped by ligation of the portal vein during hepatectomy. An auxiliary filter was provided to allow a rapid change without cessation of perfusion. Inadequate oxygenation was evidenced by a darkening of the perfusate. If the oxygen flow was adequate, this circumstance was sometimes due to a partially clogged filter. The use of the fractionating column offers a large surface area for $O_2 - CO_2$ exchange, making possible the rapid reoxygenation of the perfusate.

The vent in the hydrostatic reservoir is a necessary factor in equalizing the pressures between the air space above the hydrostatic head and that of the oxygenating column. Without this vent, blood occasionally fills the side arm, forming a continuous column of liquid which is longer than the column above the liver. This results in the formation of a siphon which decreases the hydrostatic pressure on the organ preventing adequate perfusion, and ultimately resulting in clotting and obstruction. Attempts were made initially to perfuse the livers with direct pressure from the lift pump. This did not prove satisfactory for several reasons. The pump maintains a constant rate of delivery, whereas the liver flow tends to vary. The result of this direct pressure from the pump was hemorrhage in the hepatic parenchyma and/or rupture of the portal vein. The hydrostatic reservoir is capable of compensation in its delivery rate and is, therefore, more physiologic.

Hemolysis in these experiments was minimal at the beginning of the perfusion, increasing slowly but continuously. "Plasma" hemoglobin was measured, and appropriate corrections were applied to chemically measured protein values by subtraction of hemoglobin protein. This correction factor must be applied to the estimation of plasma protein before conclusions can be made as to changes in the protein concentration.

The uptake of glycine into protein (as measured by the uptake of glycine-2-C^{14}) was 0.68×10^{-3} and 2.4×10^{-3} micromoles per gram of protein per hour into the plasma and liver proteins, respectively. It is interesting to note that both of these rates are of the same magnitude as values reported by Borsook[16]

for rabbit bone marrow activity. With bacterial contamination, C^{14} uptake into protein would be erroneously high; and a number of other chemical changes may be brought about as a result of the bacterial action. When bacterially contaminated perfusate was incubated with glycine-2-C^{14}, C^{14} incorporation into protein was 20 per cent of the total injected activity in 48 hours. Melchior and associates[15] have reported rates of methionine incorporation into protein by non-proliferation bacteria in the order of 3.0 micromoles per gram of dry weight of bacteria per hour. This rate is 10^3 times greater than that of livers calculated as dry weight of protein. This magnitude of uninhibited bacterial action emphasizes the necessity of maintaining sterility in perfusion studies.

The chemical, isotopic, and histologic results constitute conclusive evidence of a surviving organ. Obviously, these were less "healthy" livers than the same organ prior to excision. The metabolism of the hepatic cells of a perfused liver is better approximation to the normal than cells of mince or slice preparations. The conditions of the perfusion are such that the metabolic activity of the liver may be segregated from the influences of other organs which complicate studies of liver function in "intact" animals.

CONCLUSIONS

A convenient technique for the controlled study of hepatic metabolism by means of organ perfusion is presented. A relatively simple apparatus is described which can be assembled easily by other investigators.

APPENDIX

1. The upper half of the organ chamber is the dome of a vacuum distilling apparatus. It has an opening for a No. 6 rubber stopper and the ground surface has an inside diameter of 165 mm.

2. The sampling tube is gum rubber, thick-wall tubing with inside and outside diameters of ¼ inch and ¾ inch, respectively.

3. The nylon filters (Baxter Company) were from the disposable type blood recipient sets.

4. The glass valves are described by Werthessen[1] and can be purchased from MacAllister-Bicknell Company.

5. The Luer Lok adapters accompany the Brewer Automatic pipetting machine.

6. The pulsating tube consists of a glass tube of 25 by 75 mm. reduced to 6 mm. on one end. A finger cot is held in place by a Fenwal rubber cap.

7. The hydrostatic reservoir is a glass tube 40 by 100 mm. reduced to 6 mm. at the lower end. A side-arm of 15 mm. diameter is attached near the top and extends below the bottom of the reservoir.

8. The composition of the α-amino acid mixture* in grams per 100 Gm. dry weight was: L-leucine 16.5 Gm.; L-lysine 9.4 Gm.; L-methionine 4.0 Gm.; L-phenylalanine 5.3 Gm.; L-threonine 1.9 Gm.; DL-tryptophane 1.8 Gm.; L-valine 7.0 Gm.; L-glutamic acid, maximum 1.5 Gm.; and lactic acid 10 Gm.

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